

DECLARATION OF MITCHELL F. BRIN
(37 C.F.R. section 1.132)

I Mitchell F. Brin declare as follows:

1. I am a citizen of the United States and presently reside in Newport Beach, California.

2. I am over the age of twenty one, competent to testify in a court of law, and could and would testify to the matters set forth below before the United States Patent and Trademark Office, if required to do so.

3. I understand that this declaration will be used to assist prosecution of one or more of the following related patent applications:

(1) United States patent application serial number 10/933,723, including to assist to overcome a rejection in the December 15, 2006 office action in serial number 10/933,723;

(2) United States patent application serial number 10/443,593, including to assist to overcome a rejection in the November 13, 2006 office action in serial number 10/443,593;

(3) United States patent application serial number 10/726,904, including to assist to overcome a rejection in the December 15, 2006 office action in serial number 10/726,904;

(4) United States patent application serial number 10/460,898, including to assist to overcome a rejection in the November 13, 2006 office action in serial number 10/460,898, and;

(5) United States patent application serial number 10/461,829.

4. I graduated from the University of Pennsylvania in 1975 with a bachelor of arts in biology (Magna Cum Laude and Phi Beta Kappa) and I received an M.D. degree from the Columbia College of Physicians and Surgeons in 1979. I was a medical intern at the Mount Sinai Hospital and School of Medicine from 1979 to 1980. I was a neurology

resident from 1980 to 1983 at the Neurological Institute of the Presbyterian Medical Center of New York City and I was a Fellow at the Columbia University Neurological Institute from 1983 to 1986, carrying out research on the treatment of movement disorders with botulinum toxins. During the period of 1986 through 1994, I was an Assistant Professor of Clinical Neurology and Assistant Professor of Neurology at the Columbia University College of Physicians and Surgeons. At this time, I also served in the capacity of the Coordinator of the Dystonia Clinical Research Center.

5. In 1994, I joined the Mount Sinai Medical Center and School of Medicine as an Associate Professor and Director of the Movement Disorders Program where I continued my professional activities through 2000. I also served as Director of Movement Disorders and held the Bachmann-Strauss Endowed Chair in Neurology. I continue as an Adjunct Professor of Neurology at the Mount Sinai School of Medicine and became a faculty member at the University of California, Irvine, School of Medicine in 2002, as a Clinical Professor of Neurology.

6. I founded the American Academy of Neurology course on botulinum toxin, and I have organized numerous courses and symposia on botulinum toxin, and published extensively on the subject of therapeutic use of the botulinum toxins.

7. I am Founder and Past President of WE MOVE (Worldwide Education and Awareness in Movement Disorders) which is an international professional and patient educational not-for-profit organization that focuses on movement disorders. I also serve on the board of directors of the Bachmann-Strauss Dystonia and Parkinson Foundations and have served on the Board of the Exceptional Parent Foundation. I serve on the Executive Committee of the United States Pharmacopeial Convention (USP) Council of Experts, and I am the Chair of the Neurology, Otolaryngology and Ophthalmology Expert Committee of the USP. I am a member of: the American Academy of Neurology Section on Movement Disorders and Co-Founder of the Dystonia Study Group; the United States Interagency Botulism Research Coordinating

Committee, and; the University of California, Irvine, Dean of Biology's Leadership Council.

8. I have served on the editorial and peer-review boards of numerous scientific journals and on the steering committee of the World Congress on Disabilities. I am a Fellow of the American Academy of Neurology; a recipient of the FDA Commissioner's Special Citation for work with neurological movement disorders; and a recipient of the Distinguished Service and Honor Award from the American Academy of Otolaryngology-Head & Neck Surgery.

9. I was one of the first investigators to examine the use of botulinum toxin for the treatment of medical disorders and I pioneered the use of botulinum toxin for the treatment of dystonias, including blepharospasm and other debilitating neurological disorders. I have designed and conducted double blind studies examining the use of botulinum toxin for numerous conditions and have conducted numerous multicenter studies for uses of botulinum toxin.

10. Over the course of my career, I have received research funding from numerous public (e.g., National Institutes of Health, Food and Drug Administration) and private sources (e.g., not-for-profit patient Foundations, Industry grants, etc.) to further an understanding of medical disease, development of therapeutics, conduct clinical trials, and provide education about medical illnesses. Specific to the study of botulinum toxin, I have been funded by the Dystonia Medical Research Foundation, National Institutes of Health, Food and Drug Administration, Bachmann-Strauss Foundation, Allergan Inc., Athena Neurosciences, and Ipsen Pharmaceuticals. Specifically with regard to Allergan Inc., beginning in about 1990 I received several unrestricted medical grants to study various therapeutic uses of Botox® (Botulinum toxin type A purified neurotoxin complex). Additionally, between 1997 and 2000 I attended a number of Botox® advisory board and technology update meetings sponsored by Allergan and signed confidential disclosure agreements so that I could participate in these meetings.

11. Since about 1984 (including during 1993) I have diagnosed and treated many patients with botulinum toxin to treat a variety of disorders and conditions including hyperhydrosis, cervical dystonia (spasmodic torticollis), tardive dyskinesia, essential tremor, spasmodic dysphonia, smooth muscle spasm, temporal mandibular disorder, muscle spasm pain (including smooth muscle spasm pain), spasticity, swallowing disorders, and headache, including migraine headache and tension headache. I have administered different serotypes of botulinum toxin to patients.

12. A partial list of my publications is attached to this declaration as Attachment 1. These publications include Jankovic J, and Brin M., *Therapeutic Uses of Botulinum Toxin*, New Eng J. Med, 1186-1194;1991, which is cited on page 2 of each of the five pending patent applications cited in paragraph 3 above.

13. By training and experience I am an expert in the therapeutic use of the botulinum toxins.

14. I have been an employee of Allergan, Inc. of Irvine, California ("Allergan") since January 2001. My current position with Allergan is Senior Vice President, Development and Therapeutic Area Head, Botox® and Neurology. In this position I oversee the Botox® development portfolio, and direct clinical programs for uses of botulinum toxin. Allergan is the assignee of the patent applications cited in paragraph 3 above, as well as of their common parent application, U.S. application serial number 08/173,996, which has a filing date of December 28, 1993 and which is referred to hereafter as "the '996 application".

15. I have read the '996 application and in my opinion a physician of ordinary ability with knowledge of or experience using a botulinum toxin (the "Physician") would in December 1993 have very clearly realized upon reading the '996 application that the '996 patent application describes methods for treating at least hyperhydrosis, cervical dystonia, tardive dyskinesia, essential tremor, spasmodic dysphonia, smooth muscle spasm, temporal mandibular disorder, muscle spasm pain, including smooth muscle

spasm pain, spasticity, swallowing disorders, and headache (the Disorders") by administration of just the neurotoxic component of a botulinum toxin complex to a patient with one or more of the Disorders.

16. I base my opinion in paragraph 15 above on the following facts:

(1) page 3, lines 5-24 of the '996 application discloses that:

- (a) there is a neurotoxic component of a botulinum toxin;
- (b) the neurotoxic component has a molecular weight of about 150 kD;
- (c) the 150 kD neurotoxic component can be in the form of a dichain, that is comprising a 50 kD short chain and a 100 kD long chain, and;
- (d) the neurotoxic component is responsible for the toxic properties of a botulinum toxin.

(2) the matters set forth in paragraph 16(1) (a) to (d) above were also facts already established in the literature and therefore known to the Physician. See for example page 6 of Simpson L., *Current Concepts of the Mechanism of Action of Clostridial Neurotoxins*, in DasGupta B., Botulinum and Tetanus Neurotoxins, Plenum Press, New York (1993) (attached as Attachment 2), and; page 82 of Schantz E., et al., *Properties and use of botulinum toxin and other microbial neurotoxins in medicine*, Microbiol Rev 1992 Mar;56(1):80-99 (attached as Attachment 3).

(3) significantly, page 3, lines 5-24 of the '996 application discloses that use of the neurotoxic component (in either it's single or dichain forms) is "useful in the method of the present invention" thereby directly and immediately telling the Physician that the neurotoxic component can be used to treat the Disorders.

(4) on page 5, at lines 16-21 of the specification it is disclosed that a botulinum toxin is a zinc endopeptidase. The Physician would readily understand this to mean that it is the neurotoxic component of a botulinum toxin that is the zinc endopeptidase, and this is taught by the literature as well. See for example Schiavo G., et al., *Botulinum*

Neurotoxins are Zinc Proteins, J Biol Chem 1992 Nov; 267(33): 23479-83 (attached as Attachment 4).

17. It is also my opinion that the Physician upon reading the '996 application in December 1993 would have been able with little or no difficulty to obtain the neurotoxic component of a botulinum toxin so as to be able to use the neurotoxic component to treat a patient with one or more of the Disorders.

18. I base my opinion in paragraph 17 above on the following facts:

(a) the '996 application states on page 4, lines 9-12 that a botulinum toxin can be purified in accordance with known techniques. It was known to the Physician in 1993 that the neurotoxic component of a botulinum toxin could be obtained by running a botulinum toxin complex through a protein separation resin (such as a Sephadex gel) in an alkaline pH buffer. See for example Wagman J. et al., *Botulinum Type A toxin: properties of a toxic dissociation product*, Arch Biochem Biophys 1953; 45: 375-383 (attached as Attachment 5); DasGupta B., et al., *Separation of toxin and Hemagglutinin from crystalline toxin of Clostridium botulinum type A by anion exchange chromatography and determination of their dimensions by gel filtration*, J Biol Chem 1968 Mar 10; 243(5): 1065-72 (attached as Attachment 6); Schantz E., *Use of crystalline type A botulinum toxin in medical research*, being pages 143-150 of in Lewis G., *Biomedical aspects of botulism*, Academic Press, New York (1981, page 143 (attached as Attachment 7); Borodic, G., et al., *Clinical and scientific aspects of botulinum A toxin*, Ophthalm Clinics of N America 1991 Sep; 4(3): 491-503 (attached as Attachment 8), and; Schantz (1992) (Attachment 3). Thus, it was well known to the Physician, as indicated by the '996 application, that the neurotoxic component could be purified from a botulinum toxin complex

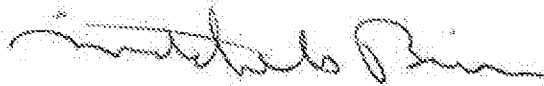
(b) additionally, it was known to the Physician that the neurotoxic component of a botulinum toxin was available for purchase simply by ordering it from a commercial supplier, such as Sigma.

(c) the '996 application gives particulars as to how a physician can administer the neurotoxic component on pages 7-8 of the specification (in the Detailed Description section of the specification) and the Physician would be aware of techniques for administration of the neurotoxic component, such as by intramuscular or subcutaneous administration.

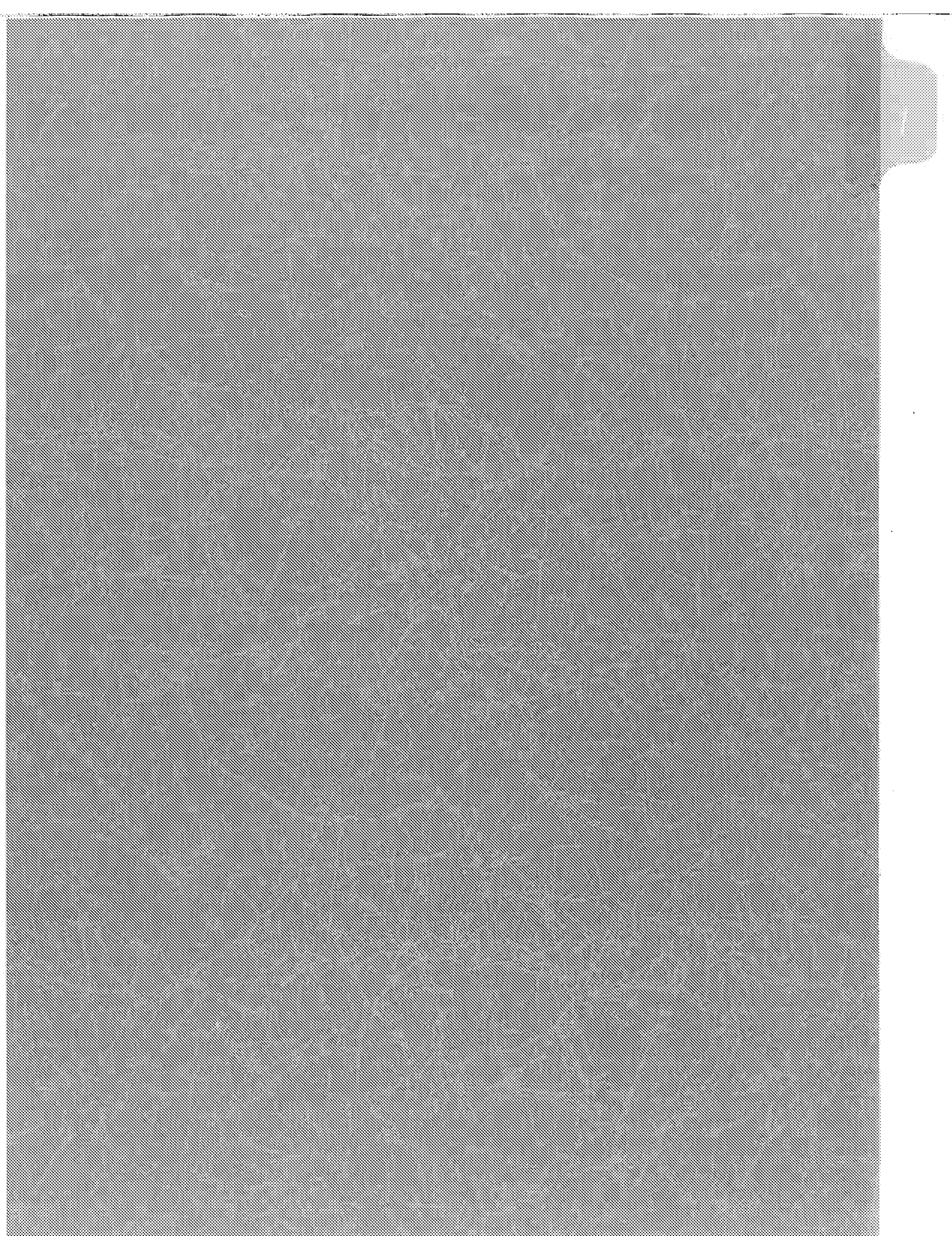
19. To reiterate, it is my opinion that the Physician after reading the '996 application would have very clearly realized that the '966 application sets forth methods for treating a patient afflicted with one or more of the Disorders using the neurotoxic component of a botulinum toxin, and that the Physician would have been able to easily proceed to treat a patient suffering from a Disorder with the neurotoxic component of a botulinum toxin based on the disclosure and guidance provided in the '996 application.

20. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity and/or enforceability of the instant patent application or any patent issuing thereon.

Executed this 28th day of March 2007 in Irvine, California.



Mitchell F. Brin



Mitchell F. Brin, M.D

PUBLICATIONS:

A. Original, Peer Reviewed Reports:

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C. Media Resource and Educational Material: Videotape and CD ROM Productions

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2. Dr. Brin is President of WE MOVE (www.wemove.org), and is contributor/editor for the entire website. This includes the content and slide series (downloadable) on dystonia, Parkinson's disease, tics/Tourettes, myoclonus, tremor and spasticity.
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BOTULINUM AND TETANUS NEUROTOXINS

*Neurotransmission and
Biomedical Aspects*

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FOREWORD

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CURRENT CONCEPTS ON THE MECHANISM OF ACTION OF CLOSTRIDIAL NEUROTOXINS

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INTRODUCTION

The purpose of this chapter is to provide a brief review of the literature on botulinum neurotoxin and tetanus toxin. This review will emphasize studies that pertain to cellular and subcellular actions of the toxins on mammalian preparations, and it will deal exclusively with issues that relate to blockade of exocytosis.

Botulinum neurotoxin and tetanus toxin act on nerve endings and other secretory cells to inhibit spontaneous and evoked mediator release.¹⁻³ The two toxins are thought to act in a somewhat similar manner, with the exception of intracellular trafficking. During the course of natural poisoning, botulinum neurotoxin binds preferentially to motor nerve endings. It is internalized by the process of receptor-mediated endocytosis, and it escapes endosomes by an acidification process. The toxin then acts locally at motor nerve endings to block acetylcholine release, and this produces flaccid paralysis. The scheme of events with tetanus toxin is somewhat different. This toxin also binds to motor nerves, and it too is internalized by receptor-mediated endocytosis, but it does not act locally. Tetanus toxin is carried by retrograde axonal transport to the central nervous system, where it exits the primary neuron, crosses the synaptic space, and preferentially binds to inhibitory nerve endings. At this point the mechanism of action of tetanus toxin is thought to be similar to that of botulinum neurotoxin. Tetanus toxin is endocytosed and released into the cytosol by an acidification mechanism. By virtue of blocking the release of inhibitory transmitters, it produces disinhibition of excitatory activity. This is believed to be the underlying basis for toxin-induced spastic paralysis.

In addition to its well-known ability to evoke spastic paralysis, tetanus toxin can also produce flaccid paralysis.² When large amounts of toxin are administered to laboratory animals, or when high concentrations are added to isolated neuromuscular preparations, some fraction of the toxin escapes retrograde axonal transport and acts locally at nerve endings to block exocytosis. In this case, the general features of botulinum neurotoxin poisoning and tetanus toxin poisoning are essentially the same.

ORIGIN AND STRUCTURE

Botulinum neurotoxin is produced mainly by *Clostridium botulinum*, but it is also made by *Clostridium baratii* and *Clostridium butyricum*. *Clostridium botulinum* synthesizes seven serotypes of toxin designated A, B, C, D, E, F and G; *Clostridium baratii* synthesizes a single serotype that is similar to type F, and *Clostridium butyricum* synthesizes a single serotype similar to type E. At the moment, *Clostridium tetani* is the only organism known to make tetanus toxin.

Regardless of origin, the synthesis and processing of botulinum neurotoxin and tetanus toxin are identical. Each neurotoxin is synthesized as a single chain polypeptide with a molecular weight of approximately 150,000. In the immediate post-translational stage the neurotoxins have diminished biological activity. When exposed to proteases, the single chain toxins are nicked to yield dichain molecules in which a light chain (Mr ~50,000) is linked by a disulfide bond to a heavy chain (Mr ~100,000). This represents the biologically active form of the toxin.

The relationship between nicking and activation has been studied in some detail.⁴ The results indicate that nicking is essential but not sufficient for full activation to occur. Several other possible sites for proteolytic cleavage have been considered, including the aminoterminal of the light chain and the aminoterminal of the heavy chain, but these appear not to be related to activation. On the other hand, Giménez, and DasGupta⁵ have obtained evidence that suggests that activation includes proteolytic cleavage at the carboxyterminus of the heavy chain.

Most of the clostridial neurotoxins have been sequenced, beginning with tetanus toxin^{6,7} and botulinum neurotoxin type A.^{8,9} The data indicate that there is significant sequence homology among the various botulinum neurotoxins, and furthermore the botulinum neurotoxins show sequence homology with tetanus toxin. A comparison of botulinum neurotoxin type A and tetanus toxin helps to illustrate the point. An overall comparison shows that there is 30-40% identity between the toxins, and when conservative changes are taken into account the identity rises to 40-50%. The greatest amount of similarity exists in the aminoterminal regions of the heavy chains, which may be the domains that promote internalization (see below).

The work on the primary structures has confirmed a belief that arose from neuropharmacologic research. The various botulinum neurotoxins and tetanus toxin are all descendants of the same ancestral parent. The primary structure work also confirms certain findings in immunology. Monoclonal antibodies have been found that recognize epitopes in several clostridial neurotoxins.¹⁰⁻¹² The commonality of epitopes is an indicator of commonality of structure.

There is much work that remains to be done on the structure of the toxins, and then to relate structure to function, but at least an elementary scheme has already evolved.¹³ It appears that the heavy chain, and more precisely the carboxyterminus of the heavy chain, plays an important role in tissue targeting the toxin. The aminoterminal of the heavy chain appears to be essential for the internalization process. The light chain acts inside mammalian cells to block exocytosis, and therefore this portion of the molecule contains the domain that poisons transmission. There is a prevailing belief that the light chain is an enzyme (viz., protease), but the substrate remains unknown.

BIOLOGICAL ACTIVITY

Definitions

As an antecedent to discussing the mechanism of action of the toxins, it will be useful to introduce the concepts of universal antagonists and differential antagonists. A universal

antagonist will be defined as a substance that blocks the action of botulinum neurotoxin by a procedure that substance is not capable of antagonizing.

The value of universal antagonists, such as binomial antagonists help to re-

Universal Antagonists

1. Binding. The binding of botulinum neurotoxin to its receptor is isolated and characterized by a variety of methods. It has been shown that work from a variety of laboratories has revealed one interaction site on the receptor. Each serotype of botulinum neurotoxin binds to a specific site on the receptor.

In spite of the apparent differences, the binding of botulinum neurotoxin to its receptor is common. Each receptor is determined by the membrane determinants that certain classes of botulinum neurotoxin complex ganglioside interaction. Free sialic acid is released from the receptor.

A more recent study has shown that the involvement of sialic acid in the binding of botulinum neurotoxin to its receptor were tested as potential antagonists. In these assays were done. In the binding of iodinated botulinum neurotoxin to its receptor were tested for their ability to compete with the binding of the iodinated toxin.

Of the various lectins tested, *Levanderia vulgaris* lectin and *Lilium* lectin were found to be the most active. The activity of toxins was tested by the release of sialic acid from N-acetyl- α -glucosamine only for N-acetyl- α -glucosamine component of the receptor (stramonium).

There were three findings: (1) There were universal antagonists of botulinum neurotoxin in diminishing the binding of botulinum neurotoxin to its receptor at high concentrations (10⁻⁶ M). (2) Lectins were very active in blocking the binding of botulinum neurotoxin to its receptor. For example, at a concentration of 10⁻⁶ M, the apparent potency of botulinum neurotoxin was reduced by 100-fold.

Findings such as these suggest that the binding of botulinum neurotoxin to its receptor is a conventional interpretation of the receptor and its function. It is advanced by Montecchi et al. that the binding of botulinum neurotoxin to its receptor is a conventional interpretation of the receptor and its function. There could be a number of reasons for this. This relatively low affinity of botulinum neurotoxin for its receptor is a consequence of the high concentration of botulinum neurotoxin in the synaptic cleft.

antagonist will be defined as a drug or procedure that delays the actions of all serotypes of botulinum neurotoxin and tetanus toxin. A differential antagonist will be defined as a drug or procedure that substantially inhibits the actions of one or several clostridial neurotoxins, but it is not capable of antagonizing all toxins.

The value of universal antagonists is that they help to clarify events that are common to all toxins, such as binding, internalization and escape from endosomes. By contrast, differential antagonists help to reveal unique characteristics of individual toxins.

Universal Antagonists

1. Binding. The receptors for botulinum neurotoxin and tetanus toxin have not been isolated and characterized (but see the promising work reported by Schiavo et al.¹⁴). However, work from a variety of laboratories on the binding of iodinated toxins to membrane preparations has revealed one interesting point. The various clostridial neurotoxins do not share the same receptor. Each serotype of botulinum neurotoxin appears to have its own receptor, as does tetanus toxin.

In spite of the apparent differences in receptors, there is one characteristic they may have in common. Each receptor may contain a sialic acid residue, or be closely associated with a membrane determinant that has sialic acid residues. This belief stems from earlier work showing that certain classes of gangliosides can cause loss of biological activity of tetanus toxin¹⁵⁻¹⁷ and botulinum neurotoxin.^{18,19} This work showed that clostridial neurotoxins had affinity for complex gangliosides, and sialic acid residues were important to the toxin-ganglioside interaction. Free sialic acid was relatively ineffective in producing loss of toxicity.

A more recent and somewhat different line of research tends to support the proposed involvement of sialic acid residues. A large number of lectins with affinity for different sugars were tested as potential antagonists of botulinum neurotoxin and tetanus toxin.²⁰ Two types of assays were done. In the first, individual lectins were tested for their ability to antagonize the binding of iodinated toxin to rat brain membrane preparations. In the second, these same lectins were tested for their abilities to antagonize the neuromuscular blocking properties of toxin.

Of the various lectins that were tested, two showed promising levels of activity. *Triticum vulgaris* lectin and *Limax flavus* lectin both antagonized the binding of toxins to membranes and the activity of toxins on isolated tissues. The lectin from *Triticum vulgaris* has affinity for N-acetyl- β -glucosamine and N-acetyl- α -sialic acid, but the lectin from *Limax flavus* has affinity only for N-acetyl- α -sialic acid. The possible involvement of N-acetyl- β -glucosamine as a component of the receptor was ruled out by testing a lectin with affinity for this sugar (*Datura stramonium*).

There were three major points that emerged from the lectin work.²⁰ First, these compounds were universal antagonists of clostridial neurotoxins. They blocked the activity of all serotypes of botulinum neurotoxin and tetanus toxin in both assays. Second, the lectins were very effective in diminishing the binding of toxins to brain membrane preparations. When tested at adequately high concentrations (e.g., 3×10^{-5} to 10^{-4} M), they virtually abolished binding. And third, the lectins were very active in diminishing the neuromuscular blocking properties of the toxins. For example, at a concentration of 3×10^{-3} M, the lectin from *Triticum vulgaris* diminished the apparent potency of botulinum neurotoxin type B by nearly two orders of magnitude.

Findings such as these tend to implicate sialic acid residues in the binding of clostridial toxins to nerve membranes. However, they do not clarify the nature of the involvement. A conventional interpretation of the data might be that the receptor is a sialoglycoprotein. Hence, preincubation of membranes with lectins that have affinity for sialic acid could lead to occlusion of the receptor and blockade of toxin binding. A more intriguing interpretation has been advanced by Montecucco.²¹ He has proposed that binding may represent a sequence of two events. There could be an initial binding step that involves a charged lipid, such as a ganglioside. This relatively low affinity binding would promote association between toxin and the plasma

membrane. This would facilitate the subsequent high affinity binding of toxin to its authentic receptor. This is a provocative idea that deserves serious consideration. In the meantime, it should be noted that lectins with affinity for sialic acid could also act as antagonists in the sequential binding model.

2. Internalization

A. Plasma Membrane. Productive internalization of clostridial neurotoxins involves a sequence of events that can be summarized as follows.¹³ Toxin molecules bind to membranes of vulnerable cells, after which they are internalized by the process of receptor-mediated endocytosis. This allows toxin to cross the plasma membrane while being retained within an endosome. Passage through the endosome membrane is thought to be initiated by the endosome itself. The membrane possesses a proton pump that progressively lowers pH within the organelle. The toxin molecule possesses a "pH sensor", and when endosomal pH falls to a critical level the sensor triggers a conformational change that exposes a hydrophobic domain that inserts into the endosome membrane. Insertion is the key event in toxin passage across the membrane, although it is not yet known how passage occurs.

The initial work to implicate a membrane penetration step was done on the neuromuscular junction. A series of studies with polyclonal antibody showed that surface-bound toxin disappeared from accessibility to extracellular antibody and that this occurred before onset of paralysis.^{1,22} These results suggested that toxin had to be internalized. Subsequent work demonstrated that several drugs known to block receptor-mediated endocytosis were toxin antagonists. The first agent to be tested was chloroquine and its analogs,²³ and this was followed by work with ammonium chloride and methylamine hydrochloride.²⁴ The results indicated that all three drugs antagonized botulinum neurotoxin, whereas ammonium chloride and methylamine hydrochloride but not chloroquine antagonized tetanus toxin. This work, in combination with the polyclonal antibody studies, was taken as evidence that clostridial toxins were internalized by receptor-mediated endocytosis.

The pharmacological work on internalization was nicely complemented by the morphological work of Black and Dolly,^{25,26} who iodinated botulinum neurotoxin and used it to do electronmicroscopic autoradiography at the neuromuscular junction. They were able to show that botulinum neurotoxin binds to the plasma membrane, and membrane-bound toxin could subsequently be localized in endosomes. Movement of toxin across plasma membranes and into endosomes was energy-dependent and accelerated by nerve stimulation. Ammonium chloride, methylamine hydrochloride and chloroquine altered uptake and/or distribution of toxin. In sum, the morphological data were very supportive of the earlier pharmacological data.

B. Endosome Membrane. The current belief is that clostridial neurotoxins possess a pH sensor that induces conformational changes that lead to exposure of a hydrophobic domain. This in turn causes the toxins to insert into the endosomal membrane and eventually penetrate it. Several lines of research support this model.

A representative study is the one conducted by Hoch et al.²⁷ on pH-induced channels in planar lipid bilayers. These workers added botulinum neurotoxin to one side of an artificial membrane, then altered pH on the cis or trans side of the membrane. They observed relatively few channels with an iso-pH of 7.0 or 4.0, but they saw rapid appearance of channels when pH on the cis-side was lowered to approximately 5.5 or below and pH on the trans-side was maintained at 7.0. Structure-function analyses revealed that the heavy chain was responsible for channel formation.

Subsequent work by Donovan and Middlebrook,²⁸ Blaustein et al.,²⁹ and Shone et al.³⁰ confirmed that botulinum neurotoxin formed pH-dependent channels in lipid bilayers. Donovan and Middlebrook²⁸ demonstrated that toxin-induced channels inserted permanently into the membrane and fluctuated between open and closed states. Blaustein et al.²⁹ and Shone et al.³⁰

extended the structure of the heavy chain.

Biochemical techniques used botulinum neurotoxin and its receptor to suggest that it was a hydrophilic ligand that achieved translocation.

Kamata et al.,³³ of cooperativity between conformational changes and the same result was obtained.

There is a close association between membranes. Indeed, botulinum neurotoxin and they showed that caused release of calcium and Rauch et al.³⁸ have shown channels in membranes.

There is evidence that a hydrophobic domain of botulinum neurotoxin,³⁹ photoactivated association with toxin. The aminoterminal half of the toxin and its colleagues have shown.

To summarize, target cells to produce a response due to receptor-mediated mechanisms that is triggered.

The putative receptor is a universal antagonist, and it possesses the ability to bind preferentially on vacuoles. It can be used to inhibit inhibiting ATPase-dependent.

Recent studies have shown that all serotypes of botulinum neurotoxin nor did it alter intracellular pH of productive internalization or acidification of endosomes.

Differential Antagonism

There are two known mechanisms and the other of which is the former and drug example of the latter.

1. Antibodies. One clostridial neurotoxin. Both the

of toxin to its authentic ion. In the meantime, it act as antagonists in the

ial neurotoxins involves cules bind to membranes ss of receptor-mediated being retained within an initiated by the endosome lowers pH within the endosomal pH falls to a s a hydrophobic domain toxin passage across the

ne on the neuromuscular at surface-bound toxin occurred before onset of lized. Subsequent work endocytosis were toxin s,²³ and this was followed The results indicated that m chloride and methyl- his work, in combination clostridial toxins were

emented by the morpho- toxin and used it to do They were able to show brane-bound toxin could isma membranes and into on. Ammonium chloride, tribution of toxin. In sum, logical data.

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t al.,²⁹ and Shone et al.³⁰ n lipid bilayers. Donovan ted permanently into the et al.²⁹ and Shone et al.³⁰

extended the structure-function analyses by localizing channel activity to the aminoterminal half of the heavy chain.

Biochemical techniques have also been used to demonstrate that pH can cause botulinum neurotoxin and its respective chains to partition into a lipid environment. Montecucco et al.^{31,32} used photoreactive reagents to monitor toxin insertion into the core of a lipid bilayer. They found that acid pH caused both the heavy and light chains to insert and be labelled. This prompted them to suggest that it was not necessary to view the heavy chain as an agent for promoting passage² of a hydrophilic light chain into the cytosol. Instead, the two chains could act cooperatively to achieve translocation.

Kamata et al.,³³ who used a fluorescent reporter group, obtained data that support the notion of cooperativity between the two chains. Their work showed that low pH induced striking conformational changes in the intact toxin leading to exposure of hydrophobic domains. The same result was obtained when the heavy and light chains were examined independently.

There is a closely related body of research that pertains to tetanus toxin insertion into membranes. Indeed, investigation of tetanus toxin began before the corresponding studies with botulinum neurotoxin. Boquet and Duflot³⁴ prepared asolectin vesicles loaded with potassium, and they showed that at low pH tetanus toxin inserted into the membrane and created a pore that caused release of cation. Borochoy-Neori et al.,³⁵ Gambale and Montal,³⁶ Menestrina et al.³⁷ and Rauch et al.³⁸ have used electrophysiologic techniques to show that tetanus toxin forms channels in membranes.

There is evidence that pH-induced changes in tetanus toxin lead to exposure of a hydrophobic domain. This was demonstrated by measuring [³H]-Triton X-100 association with toxin,³⁹ photoactivatable phospholipid association with toxin,⁴⁰ and fluorescent reporter group association with toxin.³³ Each study has demonstrated that the hydrophobic domain is in the aminoterminal half of the heavy chain and the light chain. In a related study, Cabiaux and her colleagues have shown that low pH causes tetanus toxin to induce fusion of lipid vesicles.⁴¹

To summarize, there is a consensus among workers that clostridial neurotoxins must enter target cells to produce blockade of exocytosis. Crossing of the plasma membrane is probably due to receptor-mediated endocytosis. Crossing of the endosome membrane is due to a mechanism that is triggered by a fall in pH.

The putative role of an acid-triggered mechanism has led to the discovery of another universal antagonist, a compound known as bafilomycin. This compound is microbial in origin, and it possesses the property of inhibiting membrane ATPase. Interestingly, bafilomycin acts preferentially on vacuolar ATPase rather than plasma membrane ATPase. Therefore, bafilomycin can be used to inhibit the ATPase-dependent proton pump in endosomal membranes without inhibiting ATPase-dependent ion pumps in plasma membranes.

Recent studies (Simpson, in preparation) have shown that bafilomycin is an antagonist of all serotypes of botulinum neurotoxin and tetanus toxin. The drug did not inhibit toxin binding nor did it alter intracellular expression of toxicity. Bafilomycin appeared to inhibit the process of productive internalization. This is in keeping with the action of a drug that inhibits acidification of endosomes.

Differential Antagonists

There are two known groups of differential antagonists, one of which was fully predictable and the other of which emerged quite unexpectedly. Neutralizing antibodies are an example of the former and drugs that promote acetylcholine release, such as the aminopyridines, are an example of the latter.

1. Antibodies. Although antibodies have been identified that cross-react with more than one clostridial neurotoxin (see above), no antibody has been found that neutralizes all neurotoxins. Both the linear and conformational characteristics of the active domains within

each serotype are sufficiently unique to preclude the generation of antibodies that bind to active sites in multiple serotypes. Because of this, antibodies have enjoyed little utility as research tools for probing the common structural properties of clostridial neurotoxins.

In spite of the limited value of antibodies in defining common structural elements, they may be helpful in clarifying the sequence of events in poisoning. A study with monoclonal antibodies against botulinum neurotoxin type E helps to illustrate the point.⁴² A family of monoclonal antibodies was isolated, and from this family it was possible to identify three that possessed special utility. Each of the antibodies diminished the potency of native toxin, and each interacted with a different domain in the toxin (i.e., the light chain, the aminoterminal of the heavy chain or the carboxyterminal of the heavy chain). Additionally, each of the antibodies recognized a conformational rather than a linear epitope, and it was this property that allowed them to be used as research tools.

Neuromuscular preparations were incubated with toxin under conditions that allowed binding but prevented internalization. These tissues were then exposed to one or more of the monoclonal antibodies. The results showed that monoclonal antibodies were almost as effective in diminishing the potency of bound toxin as they were in diminishing the potency of free toxin. An outcome like this reveals two interesting things about toxin binding to neuronal membranes. First, binding does not cause any of the three domains in the toxin to disappear completely from the cell surface. At least that portion of each domain that possessed the relevant epitope was still exposed. Second, binding may not be associated with major changes in the conformation of the toxin molecule. Each of the antibodies was directed against a conformational epitope, and each had substantial activity against free toxin and bound toxin. At a minimum, this means that the respective epitopes did not undergo major changes during binding.

A larger family of antibodies that recognize all epitopes in the toxin molecule would allow one to determine whether any part of the toxin undergoes conformational changes during binding. In the meantime, the data indicate that binding may be very different from internalization. As explained above, pH-induced internalization is associated with marked changes in conformation.

2. Aminopyridines. Drugs such as 4-aminopyridine and 3,4-diaminopyridine block voltage-dependent potassium channels in nerve endings. By virtue of delaying the process of repolarization, these drugs promote influx of calcium and secondarily increase efflux of acetylcholine.

The ability of these drugs to enhance stimulus-evoked release of acetylcholine led to their evaluation as possible antagonists of botulinum neurotoxin. The initial results with serotype A appeared rather favorable. The drugs slowed the rate of onset of toxin-induced paralysis of isolated neuromuscular preparations, and they even reversed - at least temporarily - the development of mild paralysis. However, the results with other serotypes were less promising. Aminopyridines provided little or no protection against most serotypes of botulinum neurotoxin and tetanus toxin.

When the actions of the aminopyridines were studied at the electrophysiological level, the differences between and among serotypes became more obvious. The disparity between responses to type A, on the one hand, and responses to type B and tetanus toxin were especially clear.⁴³⁻⁴⁵ Neuromuscular preparations poisoned with serotype A had a very low rate of spontaneous miniature endplate potentials, and the probability of evoking an endplate potential was also low. However, the addition of aminopyridines substantially increased the likelihood of evoking an endplate response. By contrast, neuromuscular preparations poisoned with botulinum neurotoxin type B or tetanus toxin showed a different result. Following addition of aminopyridines, only rarely was there an evoked endplate potential.

Closer examination of poisoned tissues appeared to reveal a reason for the disparate responses. In the case of serotype A, the combination of nerve stimulation and aminopyridines resulted in synchronous release of quanta, and thus the increased likelihood of observing an

endplate response. In desynchronized and

One especially exposed initially to serotype A exposed to serotype B added in the reverse order as well as other drugs at two sites of clostridial toxin at a site somewhat remote from the membrane, and

INTRACELLULAR

There is considerable research to demonstrate the use of antibodies to block exocytosis. This was followed by studies on poisoning.^{25,26} Next, this prompted introduction into cells of intracellular injection of transmitter release.

In addition to this highlighted an anomaly in preparations, the high exocytosis. By contrast sufficient.⁵³ There is to produce poisoning between toxin action to be explained.

Messenger Systems

The fact that there are possible targets for possible targets. Categories: i) messenger membrane. Relative to membranes as site systems and cytoskeleton.

Several laboratories messenger systems to one possible exception.

Considine and kinase C activity in an artificial agonist work raises the possibility was tested transmitter release Simpson, in preparation.

bodies that bind to active le utility as research tools ins. structural elements, they a study with monoclonal the point.⁴² A family of ible to identify three that y of native toxin, and each he aminotermius of the y, each of the antibodies his property that allowed

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endplate response. In the case of serotype B and tetanus toxin, the release of quanta was desynchronized and therefore quanta could not summate to evoke a response.

One especially interesting finding arose from dual poisoning experiments.⁴⁶ Tissues exposed initially to serotype A displayed synchronous release, but when they were subsequently exposed to serotype B or tetanus toxin the release became desynchronized. When toxins were added in the reverse order, release was always desynchronized. These data with aminopyridines, as well as other drugs, toxins and physical procedures, led to the hypothesis that there could be two sites of clostridial neurotoxin action.⁴⁶ Botulinum neurotoxin type A was postulated to act at a site somewhat remote from the membrane, whereas serotype B and tetanus toxin acted closer to the membrane, and hence closer to the site of exocytosis.

INTRACELLULAR ACTIONS

There is compelling evidence to show that clostridial neurotoxins act in the cell interior to block exocytosis. Over the period of approximately a decade there has been a progression of research to demonstrate this point. The work began with pharmacologic experiments involving the use of antibody escape and the use of drugs known to antagonize endocytosis.^{22,23,24} This was followed by studies on electronmicroscopic localization of toxin at various stages during poisoning.^{25,26} Next, it was shown that direct intracellular injection of toxin blocked exocytosis.⁴⁷ This prompted experiments in which toxin, or polypeptides derived from toxin, were introduced into cells by permeabilization techniques.⁴⁸⁻⁵¹ Most recently, it has been shown that intracellular injection of mRNA that encodes toxin leads to expression of toxin and blockade of transmitter release.⁵²

In addition to demonstrating that clostridial toxins act in the cell interior, this work has highlighted an anomaly for which there is currently no explanation. When tested in mammalian preparations, the light chains of clostridial neurotoxins are both necessary and sufficient to block exocytosis. By contrast, when tested in *Aplysia*, the light chains are necessary but not sufficient.⁵³ There is a domain in the heavy chain that is needed in addition to the light chain to produce poisoning of transmitter release. There are other structure-function differences between toxin action on mammalian preparations and *Aplysia*, and these differences have yet to be explained.

Messenger Systems

The fact that the toxins act inside nerve terminals has encouraged investigators to search for possible targets. There has been a tendency to divide these possible targets into four broad categories: i) messenger systems, ii) the cytoskeleton, iii) vesicles, and iv) the plasma membrane. Relatively little work has been done with the specific intent of evaluating vesicles or membranes as sites of toxin action. However, there is an emerging literature on messenger systems and cytoskeleton.

Several laboratories have evaluated the possibility that clostridial neurotoxins modify messenger systems that govern transmitter release. This effort has been largely negative, but one possible exception is the work on protein kinase C.

Considine and his colleagues have demonstrated that agonist-induced changes in protein kinase C activity in NG-108 cells are inhibited by tetanus toxin. This has been shown both for an artificial agonist (e.g., phorbol ester⁵⁴) and for a natural agonist (e.g., neurotensin⁵⁵). This work raises the possibility that protein kinase C may be a target for clostridial neurotoxins. This possibility was tested by determining the effects of known inhibitors of protein kinase C on transmitter release at the mammalian neuromuscular junction (Considine, Sherwin and Simpson, in preparation). Two inhibitors of the catalytic domain (H7, staurosporine) and two

inhibitors of the regulatory domain (calphostin, sphingosine) were added individually to phrenic nerve-hemidiaphragm preparations at concentrations that virtually abolished protein kinase C activity in neuronal cell cultures (e.g., NG-108). Interestingly, the tissues continued to respond for several hours even under conditions in which the enzyme was substantially or completely inhibited. Furthermore, pretreatment of tissues with inhibitors of protein kinase did not predispose them to the poisoning effects of clostridial neurotoxins.

The results with protein kinase C inhibitors give rise to three conclusions. First, the enzyme is not required to sustain short-term neuromuscular transmission. Second, protein kinase C cannot be the target for clostridial toxins. And third, protein kinase C cannot be the target for any toxin that acts rapidly to block neuromuscular transmission.

Cytoskeleton

A number of hypotheses have been advanced that implicate the cytoskeleton, and especially the actin-based cytoskeleton, in the process of transmitter release. One of the more thoroughly studied of these models is one that pertains to mediator release from adrenal chromaffin cells. According to this model, it is envisioned that storage granules are held in place by a lattice-work of actin filaments. When an agonist acts on the cell to trigger mediator release, one of its effects is to stimulate disaggregation of actin lattices. This releases storage granules that can then move toward the plasma membrane and discharge their contents.

It is unclear whether a similar model applies to cholinergic nerve endings. However, if the cytoskeleton does play a role in exocytosis, it might conceivably be the target for clostridial neurotoxins. Therefore, a series of studies have been undertaken to evaluate the possible role of actin and related molecules in cell poisoning. One line of research has focused on actin and the cholinergic neuromuscular junction⁵⁶ (Considine and Simpson, unpublished findings); a second line of research has concentrated on microtubules and central nervous system synaptosomes.⁵⁷

The role of actin in exocytosis and toxin action has been evaluated by using the botulinum binary toxin as a research tool.⁵⁸ This toxin is composed of two separate and independent polypeptide chains (Mr ~45,000 and 100,000), both of which are necessary to produce cell poisoning. The heavy chain is a tissue targeting domain that binds to receptors on vulnerable cells. The heavy and light chains of the binary toxin have no affinity for one another in solution, but when the heavy chain associates with membranes of vulnerable cells it creates a docking site for the light chain. The heavy and light chains are then internalized by the process of receptor-mediated endocytosis.⁵⁹

The light chain of the botulinum binary toxin is an enzyme that possesses mono(ADP-ribosyl)transferase activity.⁶⁰ The substrate for the enzyme is monomeric actin.⁶¹ By virtue of modifying G-actin, the binary toxin disrupts the cytoskeleton of cells. More precisely, the binary toxin exerts two specific effects. Monomeric actin is in dynamic equilibrium with filamentous or F-actin. When the binary toxin ADP-ribosylates G-actin, it irreversibly removes these molecules from the pool of monomeric actin and this in turn promotes dissociation of filamentous actin to maintain equilibrium. A related effect is that ADP-ribosylated actin acts as a capping protein, thus preventing elongation by unmodified actin.

The consequences of binary toxin action are easy to observe in cultured cells. The toxin causes flattened and varigated cells that are attached to the plating surface to retract and become round.⁶²⁻⁶⁴ In essence, the cells collapse on themselves because they lack a cytoskeleton.

The botulinum binary toxin has been used to study exocytosis in a variety of secreting cells, and the results do not encourage a belief that the actin-based cytoskeleton plays either a simple or a universal role in mediator release. For example, treatment of neuromuscular preparations with the binary toxin has no obvious effect on stimulus-evoked transmitter release. Similarly, pretreatment of cells with the binary toxin neither enhances nor inhibits the actions of clostridial neurotoxins.⁵⁶ A survey of binary toxin action on all secreting cells that have been studied to

date reveals no consistent mediator release, inhibition, or enhancement by another. These results do not support the hypothesis.

Another line of inquiry has examined the ability of clostridial neurotoxins on norepinephrine release. These drugs have little or no effect and statistically significant action is the same as that of the opposite direction. A and B and tetanus toxin.

It is encouraging to find that antagonists; microtubule inhibitors may be two classes of clostridial toxins and serotype B and tetanus toxin for this division will be

Enzymatic Actions

Although the specific reason to suspect that the structures of the toxins have zinc-containing protease activity is a vigorous search for a site during the conference, but

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date reveals no consistent action on either spontaneous or evoked mediator release.⁵⁸ Enhanced mediator release, inhibited mediator release or no effect has been observed in one cell type or another. These results do not indicate a link between actin and clostridial neurotoxins.

Another line of inquiry has been directed at microtubules. Dolly and his colleagues have examined the ability of microtubule dissociating drugs to alter the actions of clostridial neurotoxins on norepinephrine release from rat brain synaptosomes.⁵⁷ They have observed that these drugs have little or no effect on certain toxins (e.g., serotype A), but they exert a small and statistically significant effect on others (e.g., serotype B). Interestingly, this disparity of action is the same as that observed with differential antagonists such as aminopyridines, but in the opposite direction. Aminopyridines antagonize serotype A but have little action on serotype B and tetanus toxin.

It is encouraging to see that two lines of research (aminopyridines and other differential antagonists; microtubule dissociating drugs) are coming to the same apparent conclusion. There may be two classes of clostridial neurotoxins, with serotype A being a prototype of one class and serotype B and tetanus toxin being prototypes of the other. Hopefully, the molecular basis for this division will be discovered shortly.

Enzymatic Actions

Although the specific target for clostridial neurotoxins has not been identified, there is reason to suspect that the toxins are enzymes with protease activity. Work on the primary structures of the toxins has revealed that they contain a histidine motif that is characteristic of zinc-containing proteases.⁸ This observation has prompted several laboratories to undertake a vigorous search for a substrate. Some tantalizing preliminary observations were presented during the conference, but no authentic substrates for toxin action were identified.

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Properties and Use of Botulinum Toxin and Other Microbial Neurotoxins in Medicine

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INTRODUCTION

The eminent physiologist Claude Bernard wrote in his classic work entitled *Experimental Science* (8), "Poisons can be employed as a means for the destruction of life or as agents for the treatment of the sick." He went on to explain how certain toxins and poisons were valuable tools for analyses of the most delicate phenomena of living structures. Although several toxic substances of plant and animal origin were used in medical practice during his time, in recent years a great multitude of poisonous substances from plants, animals, and microorganisms are now finding use in studies on animal physiology and some are used medicinally in humans.

In December 1989 the U.S. Food and Drug Administration licensed botulinum toxin type A as an orphan drug for the treatment of the human muscle disorders strabismus, hemifacial spasm, and blepharospasm in patients 12 years of age and older, by direct injection of the toxin into the hyperactive muscle. Botulinum toxin is also being used experimen-

tally for the treatment of a number of other dystonias and movement disorders (25, 98, 191). The use of the toxin for human treatment came about over 20 years ago through the collaborative work of Alan B. Scott and E. J. Schantz. The treatment of neurological disorders with botulinum toxin type A has opened a new field of investigation on the application of the toxin to nerve and muscle tissue in the human body.

Various microbial neurotoxins are being used to understand the physiology of the nervous system and may have potential value in the treatment of certain types of muscular disorders through modification of nervous stimulation of muscle activity. Well-characterized microbial neurotoxins for this purpose include the neurotoxic proteins from *Clostridium botulinum* and *Clostridium tetani* and the low-molecular-weight neurotoxins saxitoxin and tetrodotoxin, from certain species of dinoflagellates and bacteria (Table 1). These toxins affect muscular activity by their direct action on the nervous system; for example, botulinum and tetanus toxins affect activity by a presynaptic block of the release of neurotransmitters, and saxitoxin and tetrodotoxin do so by altering the action potential at the voltage-gated sodium channels of neurons. These toxins differ from many other

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TABLE 1. Approximate relative toxicities of microbial neurotoxins

Toxin	Minimum lethal dose in mice ($\mu\text{g/kg}$)	Mol wt
Botulinum toxin, type A, crystalline	0.00003	900,000
Tetanus toxin, crystalline	0.0001	150,000
Saxitoxin (free base)	9	299
Tetrodotoxins	8-20	319

microbial toxins such as diphtheria and cholera enterotoxins in that they exhibit relatively little cytolytic or cytotoxic activity. This review describes properties of presently known neurotoxins that are obtained from microorganisms and that, through their physiological action, may be valuable in medicine and pharmacology, particularly botulinum toxin type A, the only toxin which is presently being used for the treatment and relief of several human dystonias. It also points out the need for research on methods for culturing, purification, genetic expression, and preservation of these toxins applicable to their use for human treatment.

MICROBIAL NEUROTOXINS THAT BLOCK NEUROTRANSMITTER RELEASE

Properties of Botulinum Toxin Type A Relevant to Its Use in Medicine

Developments leading to the use of the toxin for human treatment. Botulinum neurotoxins are produced by certain strains of the bacterial species *C. botulinum*, *Clostridium butyricum*, *Clostridium baratii*, and *Clostridium argentinense* (86). The toxins are classified into seven serotypes, A through G, on the basis of their immunological properties. The botulinum neurotoxins comprise a family of pharmacologically similar toxins that block acetylcholine release from peripheral nerves and cause a flaccid paralysis. All of the serotypes of toxin can poison humans and other animals, but type A has caused the severest illness and many deaths from food-borne botulism and is the best-characterized botulinum toxin. Crystalline type A toxin is the serotype that is currently being used in therapeutic applications. The following sections describe the basic properties of botulinum toxin type A and the development of the toxin as a drug.

Investigations into the use of botulinum toxin type A for the treatment of hyperactive muscle disorders originated over 20 years ago through a fortunate set of circumstances and the ingenuity of Alan B. Scott, a surgeon at the Smith-Kettlewell Eye Research Institute in San Francisco. He contacted one of us (E.J.S.) regarding the availability of a toxic substance that might be injected into a hyperactive muscle and thus serve as an alternative to surgery for the treatment of strabismus, a condition in which the eyes are out of alignment. In my research on microbial toxins I had on hand highly purified crystalline type A botulinum toxin, produced by *C. botulinum*, and saxitoxin, the potent poison produced by the dinoflagellate *Gonyaulax catenella*. The mechanisms of action of these toxins had been known for many years (29, 33, 63, 103), and their possible use in the treatment of a hyperactive muscle was apparent but had never been tested. No record of such use in animals or humans was available. Both botulinum toxin and saxitoxin cause flaccid paralysis of skeletal muscle as a result of action

on the nervous system. Botulinum toxin type A appeared to be the toxin of choice for human treatment on the basis of animal studies and accidental cases of human food poisoning in which the paralytic action on survivors lasted for many weeks whereas recovery from saxitoxin poisoning took only a few days for survivors. We therefore began our collaboration on this work by using botulinum toxin experimentally on rhesus monkeys, in which Dr. Scott surgically produced a condition similar to strabismus. With the properly determined dose of botulinum toxin injected into the more active muscle, proper alignment of the eyes was achieved.

After 10 or more years of successful experiments on monkeys, the FDA granted Dr. Scott permission to treat strabismus in human volunteers. Strabismus in humans is a disorder of vision due to turning of one or both eyes from the normal position for binocular vision and is caused by hyperactivity of one or more muscles controlling eye position. This condition in humans usually is corrected by surgery, which involves cutting away a sufficient portion of the hyperactive muscle to allow the eye to assume its normal position. Successful human treatment with the toxin involved injecting measured amounts of the toxin, under carefully controlled conditions using electromyography, directly into the hyperactive muscle pulling the eye out of alignment. Injection of botulinum toxin weakened the overactive muscle, enabling compensation by the weaker one and resulting in permanent eye alignment after a period of temporary paralysis (192). The clinical work was first reported by Scott in the 1980s (188, 189), and the properties of the toxin in relation to its use in medical treatment was reported by Schantz and Scott in 1981 (180).

Special considerations on the preparation and maintenance of botulinum toxin type A for human treatment. Although the original toxin on hand and that prepared for the monkeys was sufficient, the toxin to be used for the human trials had to be prepared under more specific conditions that would, from best judgment, meet approval by the FDA. Botulinum toxin is the first microbial protein to be used via injection for the treatment of human disease. There was no precedent for the use of a microbial toxin in this manner, and protocols for this work had to be implemented. The important considerations regarding the toxin were its purity and dose on injection. The production by culturing and the purification had to be carried out so that the toxin was not exposed to any substance that might contaminate the final product in trace amounts and cause undue reactions in the patient. These restrictions required culturing in simplified medium without the use of animal meat products and purification by procedures not involving synthetic solvents or resins. Another concern was the problem of long-term stability of the toxin so that a supply was always available. Dilution of the toxin from milligram quantities to a solution containing nanograms per milliliter presented a problem because of the rapid loss of specific toxicity on such great dilution. Toxin can be diluted in pyrogen-free water or saline if used immediately for treatment, but stabilization of the toxin for longer periods requires the presence of another protein such as gelatin or albumin (173, 177). Although the commercial botulinum type A product is prepared in the presence of human serum albumin, the use of human serum albumin presents potential problems in that certain stable viral agents carried through from donors could contaminate the toxin. These and other concerns about the preparation and use of the toxin for human treatment are reviewed and discussed in the following sections.

Mechanisms of action of botulinum toxin. The primary

structure of the neurotoxin is such that the resulting shape (secondary and tertiary structures) causes a highly specific binding and block of acetylcholine release at myoneural junctions. Botulinum toxin is toxic to all vertebrates through weakening of skeletal muscle, and death may come about through paralysis of the muscles of respiration. Van Ermen-gen (226) considered that the toxin acted on the central nervous system, but it was later shown that the action is peripheral rather than central (50, 60). Most early studies on the mechanism of action of botulinum toxin were carried out with type A crystalline toxin (29, 33, 79). Botulinum toxin blocks cholinergic transmission at all cholinergic synapses in the peripheral nervous system, but conduction along axons is not affected (79). The chemical denervation lasts for several months, and recovery of neurotransmission and muscle activity requires sprouting of new nerve endings and functional connections at motor end plates. The biochemical mechanisms of botulinum toxin in skeletal neuromuscular preparations, brain synaptosomes, chromaffin cell cultures, spinal cord cell cultures, and *Torpedo* and *Aplysia* preparations have been reviewed within the last 10 years (45, 81, 137, 158, 168, 200).

Preparation and properties of botulinum toxin type A for clinical use. The Food Research Institute, University of Wisconsin, has been involved in the production of crystalline toxin pertaining to food safety for many years, and small amounts of this toxin were used for the work on monkeys. However, the toxin that was to be used for human treatment by injection required special considerations, and preparation and purity of the toxin became essential (180). The type A toxin Hall strain was chosen for production of toxin because it consistently produced high levels of toxin (1 to 4 million mouse 50% lethal doses (MLD₅₀) per ml of culture broth). It was originally obtained from J. H. Mueller of Harvard University (118), and was developed at Fort Detrick, Md., by screening for high toxin production. Toxin is produced in a nutritive medium consisting of a casein digest, yeast extract, and dextrose at pH 7.3. Following inoculation, growth is usually complete in 24 to 36 h, at which time cells undergo lysis. Complete lysis and clearing of the culture take 2 to 3 days. The toxin is liberated during lysis and is activated by proteases present in the culture broth that convert a poorly active protoxin to the highly potent toxin.

The first successful attempt at purification of type A toxin from culture broths was accomplished by Snipe and Sommer (203) at the Hooper Foundation at the University of California in 1928, when they showed that 90% of the crude toxin could be precipitated from the spent culture fluid by the addition of acid to pH 3.5. About 20 years later, Lamanna et al. (113), starting with the precipitate, obtained the toxin in crystalline form, and then Duff et al. (59) improved the method; the improved method is the basis for the present procedure for purification. The purification of botulinum toxin type A in our laboratory for human use was designed to be carried out by the simplest procedures and avoided exposure to substances such as added enzymes or columns of synthetic resins, used in some methods, that could contaminate the preparation and be carried into the final injected preparations. It is briefly described as follows. The type A toxin in the spent broth was first precipitated by adjustment to pH 3.5 with acid; 90% of the toxin was recovered in the precipitate. The precipitate was washed with water, and the toxin was extracted with 1 M salt solution at pH 6.5 and reprecipitated with acid at pH 3.7. The toxin was extracted from this precipitate with 0.05 M sodium phosphate buffer at pH 6.8, precipitated in 15% ethanol at -5°C, redissolved in

phosphate buffer, and crystallized in 0.9 M ammonium sulfate. This simplified procedure yields, for example, 60 to 70 mg of small, white, needle-shaped crystals (0.1 to 0.2 mm in length) from a 12-liter culture (15 to 17% recovery). Recrystallization under the same conditions yields 20 to 25 mg of crystalline toxin.

The crystalline type A toxin contains 16.2% nitrogen and, as far as is known, is composed only of biologically active amino acids (32, 207) for both the neurotoxin and the nontoxic proteins. The isoelectric point of the crystalline type A toxin is pH 5.6. Under slightly acidic conditions, pH 3.5 to 6.8, the neurotoxic component of 150,000 *M_r* is bound noncovalently to the nontoxic proteins in such a manner as to preserve or help stabilize the second and tertiary structures upon which toxicity is dependent. Under slightly alkaline conditions (>pH 7.1) and in the blood and tissues of animals and humans, the neurotoxin is released from the toxin complex. RNA is also associated with the toxin complex but has no known role in activity or stability.

The molecular weight of crystalline toxin was initially shown to be 900,000 *M_r* on analysis in the ultracentrifuge at pH 3.8 to 4.4 (161, 173, 181, 229). Putnam et al. (161) showed that on electrophoresis, the crystalline toxin moved as a single substance with a molecular weight of 900,000. Lamanna et al. (115) discovered that purified type A toxin could be separated into nontoxic and toxic components, when they found that a nontoxic component precipitated erythrocytes leaving the toxin in solution. Wagman and Bateman (229) also showed that the toxin moved in the ultracentrifuge as a single substance with a sedimentation coefficient of 19S at pH 5.6, but at pH 7.3 the toxin component (neurotoxin) dissociated and moved as a much smaller molecule (7S). Later DasGupta and Boroff (46) showed that at alkaline pH the neurotoxin could be separated from the nontoxic proteins by column chromatography.

On diffusion in agar gel at pH 4.2 the crystalline toxin moves as a single substance with a coefficient (*D*) of ca. $2 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ (161, 178). However, at pH 7.3, near to the pH at which the neurotoxin and nontoxic components dissociate, the diffusion rate of the neurotoxin increased to ca. $8 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$, much higher than the rate expected for a globular protein molecule of 150,000 *M_r* (178). Diffusion depends to a great extent on the shape of the molecule, and the toxin may take on a threadlike structure that would diffuse faster than a globular structure.

The biological activity (toxicity) of the toxin, like many other biologically active proteins, is due to the spatial or conformational structure of the neurotoxin molecule (173, 182). The nontoxic proteins bound to the neurotoxin apparently play an important role in maintaining the toxic shape of the neurotoxin. Careful handling of purified toxin is therefore important for maintenance of stability. Botulinum toxin type A is readily denatured by heat at temperatures above 40°C, particularly at alkaline pH. Solutions of the toxin lose toxicity when bubbles form at the air/liquid interface causing stretching and pulling of the neurotoxin out of its toxic shape (173). This denaturation also takes place in an atmosphere of nitrogen or carbon dioxide. Dilution to extremely low concentrations (nanograms per milliliter) also tends to decrease the stability of the neurotoxin, but this can be prevented by diluting with a buffered solution (at pH 6.8 or below) containing another protein such as gelatin and certain albumins such as bovine or human serum albumin. When the pH is raised above 7.3, the neurotoxin is liberated, which is very labile. Because of its lability the neurotoxin is not practical for medical applications.

Crystalline botulinum toxin type A was the first microbial protein of this complexity to be considered an injectable substance by the FDA, and it was necessary to set down specifications for toxin quality. The following properties of the crystalline toxin obtained from many batches were found to be of the highest-quality toxin and were used for evaluation of batch 79-11, which was used in initial studies in humans and later licensed by the FDA: (i) a maximum absorbance at 278 nm when dissolved in 0.05 M sodium phosphate buffer at pH 6.8, (ii) an A_{260}/A_{278} ratio of 0.6 or less, (iii) a specific toxicity for mice of $3 \times 10^7 \pm 20\%$ MLD₅₀ per mg, and (iv) an extinction coefficient (absorbancy) of 1.65 for 1 mg of toxin per ml in a 1-cm light path.

The purity of the crystalline toxin cannot be defined strictly in terms of percent purity because of small amounts of undefined material absorbing at 260 nm, most probably nucleic acid material, which associates with the toxin during culturing and is carried through the purification and crystallization procedures. This property is peculiar to certain crystalline proteins, in contrast to the crystallization of other simpler organic substances. We have based the quality of the toxin on obtaining as low an A_{260}/A_{278} ratio as possible, near to 0.55. Toxin from the first crystallization has a ratio close to 0.6, and on the second crystallization it should be reduced to about 0.55, which is considered representative of high-quality toxin. A third crystallization may reduce the ratio slightly but at a cost of yield, because only one-third to one-half of the toxin is recovered on each crystallization. Other crystalline proteins such as human and bovine serum albumins have absorbance ratios close to 0.5 (232). If it is assumed that the absorbing material at 260 nm is nucleic acid with an extinction of 20 per mg (12 times that of the toxin), the proportion of nucleic acid in a preparation with a ratio of 0.6 would be less than 0.1%.

Another test of purity and consistency for each batch of type A toxin is the banding pattern on solution electrophoresis and gel electrophoresis with crystalline toxin and reduced crystalline toxin. At or below the isoelectric point of 5.6, the toxin moves as a single homogeneous substance, of 900,000 M_r . Toxin reduced with sulfhydryl reagents shows the distribution after electrophoresis of the nontoxic components along with the neurotoxin subunits of 100,000 and 50,000 M_r . Electrophoresis carried out on several batches of crystalline toxin showed that the toxin is judged very similar for each batch (101).

The specific toxicity of a high-quality preparation of crystalline toxin should be 3×10^7 MLD₅₀ ($\pm 20\%$) per mg. The number of milligrams for this determination is based on the A_{278} , using the extinction of 1.65 to convert to milligrams of toxin ($A_{278}/1.65 =$ milligrams of toxin [$\pm 3\%$]) (207). Because the immunological properties of type A toxin are independent of its toxic properties, the only means of evaluating the potency or acetylcholine-blocking power of the toxin is an animal assay (176). The mouse assay for toxicity determination may vary depending on the species of mice, their condition, and the conditions under which the assay is carried out. To minimize the variability, it is recommended that the mouse assay be carried out on any preparation used for human treatment with the use of a reference standard of type A toxin as described by Schantz and Kautter (177). There is no known chemical, physical, biological, or immunological test available that can replace the mouse test for toxicity evaluation.

An important factor in the medical use of botulinum toxin is a method of storage for retention of toxicity. The crystalline toxin formerly provided for reference in food assays was

dissolved and stored in 0.05 M sodium acetate buffer (pH 4.2) at 4°C (177), in which it retained toxicity for 1 to 2 years before a significant loss (20%) could be detected by mouse assay. The difficulty with storing the toxin in acetate buffer is that freezing causes complete detoxification and reliance on storage at 4°C without danger of freezing is not practical under certain circumstances. However, we have found that the most satisfactory method of storage is to leave the crystalline toxin at 4°C in the mother liquor of the second crystallization, in which toxicity was retained for 10 or more years. Retaining stability is important because it makes available a bulk supply of toxin to draw from over an extended period. When the FDA approved experimental trials on human volunteers, a large batch of crystalline toxin was prepared in November 1979 (designated 79-11) expressly for the human trials; 100 mg was supplied to Alan Scott and 50 mg was retained in storage at the Food Research Institute. This batch has been the sole source of botulinum toxin type A accepted by the FDA for human treatment and has been used by many physicians throughout the United States and some foreign countries. However, some loss in toxicity has occurred in batch 79-11, and we recommend that fresher batches of toxin periodically be prepared to avoid detrimental changes that may occur on aging. Crystalline type A toxin prepared in our laboratory does not appear to differ in potency or clinical efficacy from type A toxin prepared in England by using anion-exchange chromatography and RNase treatment (133, 222). However, we do not recommend the use of methods of purification involving enzymes, various exchangers, or synthetic solvents because of the chance of contamination.

Preparation of the toxin for dispensing as a drug and compatible for injection into muscle required (i) dilution in a suitable medium for stability of toxicity, (ii) filtration for sterility, and (iii) drying. Diluting a solution of botulinum toxin type A from a concentration of 1 or 2 mg/ml to nanogram concentrations causes detoxification unless another protein is added for protection. Gelatin at 2 to 3 mg/ml is generally used at pH 6.2 in the standard procedure for the mouse assay for toxin in foods (177). Bovine serum albumin has been used at 2 to 3 mg/ml in acetate buffer at pH 4.2 for good stability (177), and human serum albumin was adopted for medical use. Filtration in the presence of additional protein can be carried out successfully to remove bacterial contamination without loss of toxicity. However, drying, which would have many advantages in long-term stability, under the conditions with human serum albumin at pH 7.3 resulted in a substantial loss (50 to 90%) of toxicity. This loss of toxicity is a very important consideration because of the possibility that the inactivated toxin will form a toxoid and immunize the patient against the toxin on continued use. Various methods of drying, particularly lyophilization, resulted in such losses. Experience with the toxin has proved that stability of toxicity is dependent on low pH (<7), but such low pHs are not compatible with injections into muscle tissue. A significant problem is the development of a medium and conditions to overcome the losses on drying, and research for this purpose is being carried out in our laboratory.

Therapeutic applications of botulinum toxin. Clinical studies have indicated that toxin injections can provide profound symptomatic relief for humans suffering from a wide variety of disorders characterized by involuntary movements of muscle groups (Table 2), particularly those involved in focal or segmental dystonias (25, 64, 92, 127). In 1911, Oppenheim (151) introduced the term "dystonia musculorum defor-

TABLE 2. Focal dystonias and involuntary movement disorders successfully treated with botulinum toxin type A^a

Condition	Symptoms of disease
Strabismus.....	Crossed eyes
Blepharospasm.....	Spasmodic eye closure
Hemifacial spasm.....	Facial twitching and spasms
Eyelid disorders.....	Inward turning of eyelid
Spasmodic torticollis.....	Abnormal movements or twisting of the neck and head
Oromandibular and lingual dystonia.....	Sustained mouth closure or lingual muscle contractions
Focal dystonias of the hand.....	Writer's cramp, musician's cramp, hand and arm muscle spasms
Spasmodic dysphonia.....	Uncontrolled vocal fold spasms
Other voice disorders.....	Vocal tremor, stuttering
Neurogenic bladder.....	Abnormal urinary control; results from spinal cord injury
Anismus.....	Uncontrollable anal sphincter contraction
Limb spasticity.....	Occurs following stroke and other neurological disorders including cerebral palsy

^a Listed approximately in decreasing order of numbers of patients treated (25, 98, 179, 191).

mans" to describe children who had movement disorders such as twisted postures, bizarre walking with bending and twisting of the torso, and severe muscle spasms. Oppenheim pointed out that progression of symptoms often resulted in fixed postural deformities. Dystonia is currently defined as "a syndrome of sustained muscle contractions, frequently causing twisting and repetitive movements or abnormal postures" (64, 127). Dystonia can affect all regions of the body (127). Many patients with dystonias have been diagnosed as experiencing psychological stress and referred for psychological therapy (64), but were later found to suffer from specific neurological diseases (64, 127). Adult onset of focal or segmental dystonias (which affect only one or a few muscle groups) are more common than generalized dystonias (64, 127). A study in Minnesota estimated the prevalence of various dystonias to be 391 per million population (147). Focal dystonias may spread and lead to generalized dystonias, in which several muscle groups are involved. Focal dystonias progressed to generalized conditions in nearly 60% of affected children (onset before age 13) and in about 3% of adults (onset after age 20) (127).

Crystalline botulinum toxin has had great benefit in the treatment of involuntary muscle conditions, and injection of toxin is now considered the most effective treatment for a variety of focal dystonias (25, 98, 191). On injection the toxin acts directly or indirectly to alleviate conditions that result from muscle hyperactivity. Direct paralysis of target muscles is desired for certain indications including blepharospasm, torticollis, and other focal dystonias. Depending on the syndrome, toxin injection generally relieves undesired muscle movement for a few months, after which the abnormal movement returns and repeated injections are required. Paralysis of certain muscle groups can also lead to secondary desired effects (191). For instance, paralysis of a hyperactive muscle enables compensation by a weaker muscle, as in treatment of strabismus and certain limb muscle spasmodic disorders. In these conditions, the balancing of agonist and antagonistic muscle systems is the desired effect (191).

Strabismus was the first syndrome for which botulinum toxin therapy was introduced as an alternative to surgery

(188, 189, 192). Botulinum toxin is usually injected into the recti muscles with a Teflon-coated needle and electromyographic guidance to ensure accurate placement in the muscle; this is usually an office procedure. The toxin evokes a temporary denervation and muscle weakening, allowing the globe to return to normal alignment. Although botulinum toxin will not replace conventional surgical treatment, it has proved to be a useful adjunct to surgery in certain cases (189, 191).

Botulinum toxin is being used primarily for the correction of focal dystonias and other regional movement disorders. One syndrome approved for treatment is essential blepharospasm, in which persons suffer from involuntary eyelid closure. Blepharospasm is often accompanied by involuntary movements of head and neck muscles, a condition known as Meige syndrome (98). Meige syndrome manifests as uncontrolled blinking (blepharospasm) plus involuntary facial grimacing, frowning, facial contortions, spasmodic speech, and neck pulling (spasmodic torticollis) (24, 25). The age of onset of blepharospasm is often 50 to 70 years, and the syndrome may progress to other muscle regions. Injections of botulinum toxin type A into the orbicularis oculi muscle has given clinically significant benefit in 70 to 90% of more than 8,000 treatments (98). In most patients, the latency period from injection to onset of improvement was 2 to 5 days and relief persisted for an average of 3.5 months. The average dose was ca. 20 U (191). In some treatments, toxin diffused to neighboring muscles and caused temporary ptosis. Some patients have received repeated injections for 7 years or more, and no adverse long-term effects have been observed.

Hemifacial spasm is an often disfiguring syndrome characterized by involuntary movement of facial muscles controlled by the seventh facial nerve. Patients often find the movements disfiguring and socially and functionally incapacitating (25). Treatments with neuroleptic medications have been entirely ineffective. Injection of botulinum toxin (generally 10 to 20 U) has relieved hemifacial spasm in more than 90% of the patients treated. Most patients experience relief for 3 to 4 months, after which repeated injections have provided long-term relief in most individuals.

Spasmodic torticollis (cervical dystonia) is a dystonia affecting neck muscles and causing the head to involuntarily deviate in any direction (25, 75, 98, 208). It is among the most common dystonias, and the spasmodic contractions can cause posture deformity, head tremors, and pain. Over 1,000 cases of spasmodic torticollis have been treated, and the studies have reported improvement in 50 to 90% of the patients, depending on the dose and placement of the toxin. Comparatively large doses of botulinum toxin are used for injection at multiple sites. The larger quantities of toxin can diffuse to neighboring muscles, causing ptosis and other side effects.

Certain other diseases involving involuntary muscle movements have been successfully treated with botulinum toxin in a limited number of patients (reviewed in references 25, 98, 191, and 218). These include writer's and musician's cramps, hand tremors, spasmodic dysphonia and other laryngeal dystonias, neurogenic bladder as a result of spinal cord injury, spasms of the rectal sphincter (anismus), limb muscle spasms following stroke, leg spasms from multiple sclerosis, and spasticity in children with cerebral palsy. Botulinum toxin could potentially benefit humans who suffer from a variety of other hyperkinetic movement and muscle tone disorders including tics, tremors, bruxism, and pain brought on by muscle spasms (25, 98, 99, 218).

Although botulinum toxin is currently used for treatment of regional muscle groups, limited success has also been achieved with patients who suffer from hyperactivity of several muscle groups. Botulinum toxin has found limited use in tardive dyskinesic syndrome (221), a chorea marked by irregular dystonic movements and postures that can develop in mentally ill patients after treatment with neuroleptic medications. Some of these patients experience marked distress and suffer from disparate spasmodic disorders including repetitive blinking, backward arching of the head and trunk (retrocollis), rocking of the body, mouth grinding (bruxism), and involuntary voice sounds and grunting. In a pilot study, four patients were injected in diverse muscles and marked improvement was found in 2 weeks in all four individuals. Not all movement disorders in these patients improved, but several did including retrocollis, mouth control, and bruxism. Treatment of tardive dyskinesic syndrome by chemical denervation with botulinum toxin is complex because it involves different muscle groups. The strategy has been to focus toxin injection on the most involved muscle groups.

Generalized dystonias such as those observed in Parkinsonism present difficult problems for treatment because of the many muscles involved, but it is possible that if a proper method of administration could be worked out, these generalized conditions could be treated with toxin. One possible but untried route is the administration of low intravenous doses by which the toxin would spread regionally to many muscles.

Side effects of botulinum toxin. No adverse clinical effects of botulinum toxin have been found in patients who received low doses of botulinum toxin, e.g., ≤ 20 U. Single-fiber electromyography analysis has shown that injection of relatively large quantities of botulinum toxin (140 to 165 U) leads to toxin spread, weakening of distant muscles, and uncharacterized subclinical effects (116).

The primary side effect associated with local injections of botulinum toxin is weakening and ptosis of nearby muscles. One of the most prevalent and disturbing side effects is dysphagia, or the inability to swallow, and several patients have experienced upper airway obstruction after treatment with relatively high doses (>150 U) of botulinum toxin (25, 208). Dysphagia may be related to generalized weakness and inability to hold the head erect (75) or to weakening of muscles involved in swallowing. It may also be related to the dose and injection strategy used. To prevent dysphagia, Borodic et al. (23, 24) have recommended, on the basis of studies of toxin diffusion in tissues, the use of ≤ 100 U per treatment injected into several sites. Further research is needed to identify the lowest dose of toxin and sites of injections that will produce the desired control and prevent migration of toxin to neighboring muscle groups. Local side effects could be increased in patients who are being treated with drugs other than botulinum toxin that affect neuromuscular transmission (4).

There is interest among physicians in developing methods to prevent the spread of toxin to neighboring muscles. Scott (190) demonstrated that injection of antitoxin at the correct time following toxin injection partially prevented toxin migration. The currently available equine antitoxin could lead to undesirable reactions in some patients, and it would be valuable to have a source of human antibodies. In January 1991, human immunoglobulin G pooled from immunized human volunteers became available in a phase II clinical trial by the Orphan Drug Program of the FDA as a potential treatment for infant botulism (70). A similar pool of human

antibodies could also be useful to alleviate side effects of botulinum toxin injections without leading to patient reaction to the antiserum.

Changes in muscle tissue following botulinum toxin type A injections. Changes in skeletal muscles after botulinum toxin type A injection have been studied in animal models (53, 56, 57, 155). Duchen (56, 57) found that muscle fibers became atrophied and sprouting of nerve fibers was induced after injection of toxin into the leg muscle of mice. Sprouting of motor nerves was observed after 6 to 7 days and progressed for several weeks in the red soleus muscle; it occurred later in the predominantly white gastrocnemius muscle. Nerve sprouting occurred as complex branched arrangements which were apparently unable to establish functional connections for several weeks. The muscle fibers atrophied for 6 weeks or more and then increased in diameter to within normal limits within a few weeks. Changes in the localization and intensity of cholinesterase staining reflected the morphological changes. This work was important because it provided a new approach to quantitative characterization of reinnervation of denervated muscle. Pestronk and Drachman (155) evaluated motor nerve sprouting quantitatively after presynaptic blockade with botulinum toxin by measuring acetylcholine receptors with ^{125}I -labeled α -bungarotoxin. Muscle disuse was maintained by repeated injections of tetrodotoxin. They showed that the amount of sprouting was correlated with the number of acetylcholine receptors and was greatest in the botulinum-poisoned muscles. Sprouting was inhibited by α -bungarotoxin, suggesting that the acetylcholine receptors had an important role in inducing sprouting and muscle reinnervation. These results suggest that the use of a combination of botulinum toxin and α -bungarotoxin could prolong muscle paralysis.

In an approach derived from that of Duchen (56, 57), Borodic et al. (23–26) have used the albino rabbit as an animal model to quantitatively determine toxin spread from the site of botulinum toxin injection. Acetylcholinesterase staining, muscle fiber size analysis, and ATPase staining were used to establish a denervation gradient. A gradient effect up to 30 mm from the site of injection of 2 to 3 U of botulinum toxin type A per kg was found with respect to morphological changes in muscle fiber size and histological staining. At distances greater than 30 mm, there was substantially decreased staining and much less muscle atrophy. Very similar results were found in a study with crude type B toxin (26). The denervation indicated by histochemical staining and fiber size analysis appeared transient and lasted for about 3 months for both type A and B toxins. By using muscle biopsies, innervation sites were also determined with humans (23–25). Borodic et al. (23) have also used electrical stimulation to determine motor points and optimal injection sites in botulinum toxin therapy.

Immunity to botulinum toxin. There is considerable concern about the possibility that patients will develop antibodies and become refractory to botulinum toxin treatment, particularly when relatively high levels of botulinum toxin are injected repeatedly over several years. The dose of toxin required to trigger antibody formation in humans is not known. The minute quantities of toxin ingested in food-borne botulism are not sufficient to evoke antibodies. Recurrent episodes of type B and type E botulism have been documented in the same individual, supporting the notion that repeated exposure to botulinum toxin may not impart long-term immunity (6, 186). Repeated sensitivity to tetanus toxin in humans has also been reported (34).

Toxoid is commonly injected into laboratory workers to

stimulate antibodies and protect against accidents. The minimum dose of toxoid to elicit immunity in humans varies greatly with the individual and the toxoid preparation (3, 80, 197), but is probably similar to the immunological response to tetanus toxoid (73). Repeated injections of botulinum pentavalent toxoid after 0, 2, and 12 weeks and yearly boosters gave final titers of 3.2 IU of anti-A antibodies, 0.4 IU of anti-B antibodies, and 2.5 IU of anti-E antibodies per ml in a man (80). Antibodies were slow to develop, and a steep rise in the level of anti-A antibodies occurred in the fourth year of immunization. In an investigation of 77 patients subjected to the current U.S. schedule of toxoid injection at 0, 2, and 12 weeks, Siegel (197) reported that neutralizing antibodies to type A and B toxins were low or absent after the 12-week shot and significant titers were present only after yearly boosters. After the first booster, 74 (96%) had an anti-A antibody titer of 0.25 IU/ml or more, and only 44 (57%) of the subjects had an anti-B antibody titer of 0.25 IU/ml or more. (1 IU is defined as the amount of antibody neutralizing 10,000 MLD₅₀s.)

Antibody formation has been observed in a small number of patients injected with botulinum toxin (98, 191). To date, about 12 of more than 7,000 patients treated have developed antibodies to type A botulinum toxin. Six patients injected with 300 to 400 ng and one injected with repeated 100-ng doses within 30 days developed antibodies within 30 days (191). Antibodies have been demonstrated to reduce the beneficial effect of treatment (98). More work is needed to evaluate the incidence of antibody formation and other immunities in patients repeatedly treated with toxin over several years.

Properties and Uses of Serotypes of Botulinum Toxin Other than Type A

Seven known serotypes of botulinum toxin (A through G) have been isolated and characterized (213), and it is likely that types other than type A will be used clinically, particularly in patients who develop immunity to type A. Furthermore, evidence is accumulating to show that different types bind to different acceptors and may have subtle differences in their mode of action and that they could therefore complement type A in clinical applications. In the following sections, we review various basic science aspects of the botulinum toxins, especially as they pertain to potential clinical applications.

Botulism in humans. When botulinum toxin enters the circulation from contaminated food or infection, it can cause a severe paralytic disease. Types A, B, and E have most commonly been involved in human botulism (168, 213, 215), and type F has been the causative type in at least two outbreaks of food poisoning (78). Symptoms and severity of botulism differ depending on the serotype and amount of toxin ingested, suggesting possible differences in the mechanisms of intoxication (215). Clinical observations have indicated that type A food-borne botulism is often more severe and associated with higher mortality than botulism from other types (37, 52, 95). A rapid onset of neurologic signs indicates a more severe episode of the disease (38). Benign forms of botulism in which the course of the illness is milder and longer lasting have also been reported, particularly for type B (43, 100, 109, 209).

Botulism in humans generally manifests as a rapidly progressive symmetrical neuromuscular paralysis. Patients with botulism generally stay mentally alert during the poisoning unless anoxia sets in (108). Sudden respiratory or

cardiac arrests and airway obstruction, leading to death, can occur (109). Cardiac effects of botulinum toxin in animals and in humans have been reported (114, 215).

Botulinum toxin most often initially affects eye muscles supplied by susceptible cranial nerves, and the first signs of botulism are often blurred and double vision (215). As the paralysis progresses and peripheral nerves are affected, signs such as dry mouth, difficulty in swallowing, weakness in head and neck movements, and difficulty in breathing become apparent. In type A and B botulism, loss of musculature control manifests as ptosis and drooping of eye muscles, hypoactive gag reflex, and weakness in upper and lower extremities (95). Atypical symptoms including asymmetric or late-onset of neurologic signs, paresthesia, nystagmus, ataxia, and sensory abnormalities are not uncommon (31, 95, 215). Ingested botulinum toxin can paralyze all muscles of the body. Symptoms of botulism sometimes last for months, and recovery requires reinnervation by new nerve terminal axons and end plates. Weakness and fatigability may persist for 1 to 2 years (215). Recovery in adults is generally complete (38), but there are reports of central nervous system involvement in infant botulism (100a).

Since botulism is rarely encountered, it can be difficult to diagnose rapidly. Electromyography is useful for detection of decreased amplitude of muscle action potential in weakened muscle (215), and since conduction along the nerve axons is not altered by botulinum toxin, the proximal motor nerve conduction rates and distal latencies are normal (42). Botulism is confirmed by the demonstration of botulinum toxin in the patient's serum or stool or in suspect food by mouse assay and neutralization with type-specific antitoxin (52). Botulinum toxin has been found more often in the serum of patients with type E or B botulism than with type A, possibly because of the greater affinity of type A toxin for tissue acceptors.

The actual dose of botulinum toxin to cause food-borne intoxication in humans is debatable and depends on the individual, the source and type of toxin, and the amount ingested. Accidental cases of human botulism from toxin-contaminated food showed symptoms of botulism and occasionally death from as little as 0.1 to 1 µg (100 to 1,000 ng or 3,000 to 30,000 MLD₅₀s) (134, 140, 183), but results were quite variable, probably because of individual variation in the amount absorbed and the stability of the toxin in the gut. More data on toxicity is available for lower animals and monkeys. The lethal dose of crystalline toxin type A in mice was 1.2 to 2.5 ng (0.03 to 0.07 U)/kg (76, 80) and was 0.5 to 0.6 ng/kg for guinea pigs and rabbits (76). Scott and Suzuki (193) determined that the intramuscular LD₅₀ for juvenile monkeys (*Macaca fascicularis*) was ca. 39 U/kg (ca. 1.25 ng/kg) of body weight. Herrero et al. (91) reported a similar lethal dose of 40 U/kg by intravenous injection in *Macaca rhesus*. In Gill's table of lethal amounts of bacterial toxins, he reported botulinum toxin to be the most potent toxin known for primates, the lethal quantity of type A toxin being 0.5 to 0.7 ng/kg of body weight for monkeys and ca. 1 ng (30 U)/kg for humans (76). Larger quantities of types C₁, D, and E may be required to cause death in monkeys, whereas less type B is required (76). No data on intravenous toxicity are available for humans for botulinum toxins, but humans are probably as sensitive as guinea pigs and would be expected to be about as sensitive as monkeys.

Toxin production by the various serotypes of *C. botulinum*. Use of the various types of botulinum toxin in medicine will require a plentiful source of the toxins. The production of type A toxin under controlled conditions by the Hall strain,

as used for the preparation of toxin for human treatment, gives a uniform-crystalline toxin in high yields. The toxin complexes of the other types have also been obtained by culturing and purification and could be useful clinically. However, the strain, medium composition, and culture conditions affect the yields and structures of the botulinum toxins.

To obtain the greatest quantity and highest quality of toxin, it is essential to maintain strains of *C. botulinum* that consistently produce high levels of toxin. However, the bacterium has a frustrating tendency under laboratory conditions to gradually lose its ability to produce high levels of toxin. Lewis and Hill (118) reported that the Hall strain made decreasing quantities of toxin on successive subcultures. Huhtanen (96) also reported that strains of type A and B toxins frequently become nontoxigenic during culture. A more complete understanding of the physiological and genetic factors that control toxin production will be valuable for the development of other types.

The highest levels of toxin in group I *C. botulinum* (proteolytic strains of types A, B, and F) are generally produced in cell populations that undergo rapid autolysis and do not sporulate (21, 27), although Siegel and Metzger (198) obtained titers of 6.3×10^5 U with the Hall strain in a fermentor without appreciable cell lysis. Toxin formation is poor during sporulation, and spores contain only small quantities of toxin (ca. 1% of that found in cytoplasm) (58, 77). Takumi et al. (216) reported the isolation of nontoxigenic variants of *C. botulinum* type A that had enhanced sporulation. The strain used for production of type A, the Hall strain, sporulates very poorly. Therefore, encouraging vegetative growth and autolysis and discouraging spore formation may be important for obtaining good yields of toxin.

Toxin formation is controlled by nutrition in group I and II *C. botulinum* (119, 152). Arginine delayed autolysis, affected sporulation, and repressed toxin formation in group I *C. botulinum* (28, 154). Toxin formation was repressed about 10,000-fold in group I, including the Hall A and Okra B strains, when abundant arginine was available in the medium (152), probably owing to nitrogen repression of toxin gene expression. Protease was also decreased by arginine in group I *C. botulinum*. In group II *C. botulinum* (nonproteolytic strains of serotypes B, E, and F), tryptophan availability repressed toxin formation, probably also in response to nitrogen sufficiency (119). These results indicate that fermentation conditions and mutant strains could be developed for improved toxin production.

Significance of complexes on toxin quality. The strain and culturing conditions also affect the quality of toxin that is produced. Schantz and Spero (181) found that botulinum toxins of the different serotypes occur in spent cultures as large protein complexes. In the ultracentrifuge the sedimentation coefficients for the complexes were 19S for type A, 16S for type B, and 13S for types C, D, E, and F. Sugii and Sakaguchi (211) showed that high-molecular-weight toxin complexes occur naturally in foods. It is now known that each of the types of botulinum toxin produced in food or in culture are conjugated proteins ranging in molecular weight from 300,000 to 900,000, comprising a molecule made up of one or two neurotoxic units of about 150,000 M_r , noncovalently conjugated to nontoxic proteins (168, 181, 213).

The formation of toxin complexes is very important for use of the toxins in medicine because the nontoxic proteins play an important role in maintaining the stability of the neurotoxic units. Isolated neurotoxic units were poorly toxic

to mice when administered orally (169, 170, 210). Peroral toxicity increased with incremental association of the neurotoxins with the protective proteins (150, 168–170, 210). The larger (19S and 16S) complexes of botulinum toxin types A and B were more toxic by the oral route and more resistant to acid and pepsin than were the smaller complexes. The isolated neurotoxins were rapidly inactivated by these conditions. Variations in the toxigenicities of different strains also probably depend on differences in the structures of the complexes. Ohishi (150) found that the oral toxicities differed considerably for the toxins of certain type A and B strains of *C. botulinum*. Of five B strains, Okra B produced the most potent toxin by oral challenge in mice. The 16S complex of the toxin was 700 times more potent than the 16S molecule from strain NH-2. A hybrid composed of the neurotoxin from NH-2 and the nontoxic components from Okra increased the oral toxicity close to that of the native Okra toxin, probably by protection of the neurotoxin in the gastric and intestinal tracts.

The size of the complex formed in types A, B, E, and F depends on the medium for bacterial growth. It has been known for years that some foods such as vegetables have high botulinogenic properties (134, 212). Sugii and Sakaguchi (212) showed that type A and B *C. botulinum* produced the stable 19S and 16S high-molecular-weight complexes in vegetables, whereas they produced the less stable 12S complex in tuna and pork. Nonconjugated neurotoxin was not found in any of the food substrates. They found that addition of iron or manganese to the growth medium resulted in a higher concentration of small toxin complexes (12S and 16S) in type A *C. botulinum*, suggesting an influence of metals on the size and stability of the complexes.

Biochemical and genetic properties of the neurotoxin component. The biochemistry of purified botulinum neurotoxins, particularly type A toxin, has been studied in considerable detail, and authoritative reviews are available (45, 81, 213). Neurotoxins have been purified for all serotypes except for type G; the type G toxin has been purified to a protein complex of high toxicity, but further purification resulted in substantial loss of toxicity (126, 146). Within a given type of toxin and strain of producing bacterium, there may be considerable heterogeneity in molecular structure and antigenicity, giving a mosaic structure (139). The neurotoxins all have high specific toxicities, from 10^7 to 10^8 MLD₅₀/mg of protein (213).

All types of neurotoxins are synthesized as single-chain protein molecules of about 150,000 M_r with low toxicity. The protoxins are released from the bacterium during culture (48). Those of proteolytic (group I) *C. botulinum* strains are cleaved by extracellular proteases into two-chain molecules consisting of an H (heavy) subunit of about 100,000 M_r and a L (light) subunit of about 50,000 M_r (45, 47, 48, 171, 196). Toxin preparations from nonproteolytic cultures require exogenous protease treatment for protoxin activation. The H and L chains are covalently linked by at least one disulfide and noncovalent bonds (45) and possibly a metal component (10, 11). The H and L chains of the neurotoxins can be separated by chromatography after treatment with dithiothreitol and urea (171). The isolated chains are not toxic by themselves but can be recombined under carefully controlled conditions to obtain active toxin (110, 123, 214, 230). Recently a chimeric toxin which retained considerable activity was prepared between the L chain of tetanus toxin and the H chain of botulinum toxin type A (230). Chimeric toxins composed of defined fragments, e.g., the H chain from botulinum toxin and the L chain from ricin, could be

valuable in medicine, but much work needs to be done on their formation and clinical testing.

During proteolytic cleavage the neurotoxins undergo a molecular change in shape that increases toxicity (48). The nicking region was recently reported to contain multiple target sites susceptible to more than one protease (45, 47). DasGupta and Dekleva have proposed that two peptide bonds in a short region are cleaved at different rates during maturation of type A toxin and that 10 amino acids are excised (45, 47). The control of proteolysis to increase stability could be useful in the preparation of the toxins for medicine, as has been achieved with tetanus toxin, and to obtain defined fragments for construction of toxins with desired properties.

The presence of metals in neurotoxins may affect their stability. Bhattacharyya and Sugiyama (10, 11) reported that chelators for iron and manganese inactivated purified type A botulinum toxin and tetanus toxin. Analysis of purified botulinum neurotoxin for metal content by neutron activation indicated that one atom of iron was present for each toxin molecule. It was suggested that metals may be involved in linkage of the H and L chains of botulinum and tetanus neurotoxins (10, 11). Kindler and Mager (107) found that metal availability in the culture medium affected the formation of toxin. Culturing *C. botulinum* in a medium containing EDTA did not inhibit growth but completely suppressed toxin formation. The biological activity of botulinum toxin may depend on a transition metal component, possibly Fe. The presence of metals could be important in maintenance of activity and protection from oxidation during drying and for long-term stability.

Recent genetic advances have increased our understanding of the structure and expression of the botulinum toxins. The genes coding for botulinum neurotoxin types A, B, and E are present in one copy on the chromosome in representative strains (14, 219). Genomic libraries of *C. botulinum* type A chromosomal DNA (strain 62A or NCTC 2916) were prepared on plasmids and transformed into *Escherichia coli*. For safety reasons, separate subfragments that were 2 kb or less in size and did not encode the entire neurotoxin gene were cloned. Open reading frames which encoded a sequence corresponding to a polypeptide of 1,296 amino acid residues, 149,425 M_r (14) or 149,502 M_r (219), were identified. The nucleotide sequences were in agreement with the partial nucleotide sequence reported by Betley et al. (9). The promoter of the BoNT/A gene was not transcribed in *E. coli*; this may have been caused by the frequent presence of codons in the promoter region that are not normally present in *E. coli*. Codon usage in the botulinum toxin gene was similar to that previously found for the tetanus toxin gene (61, 65, 66). Overall, 90.3% of the degenerate codons ended in A or U. An exception to the codon bias occurred for Lys codons, in which the frequency of AAA and AAG was nearly the same (24 AAA and 20 AAG) (219) compared with 98 AAA and 9 AAG for the tetanus toxin gene. In *C. botulinum*, AUG and UAA were translational initiation and termination codons, respectively, and strong bias was found for Arg and Ser codons. Binz et al. (14) found that the A+T content in the 5'-noncoding region of the type A and type E toxin genes was 80.4 and 80.3%, respectively, higher than in the coding regions, where 73.6 and 72.1% A+T were found. Examination of the upstream region indicated that transcription started 118 to 127 nucleotides upstream from the translation initiation site (14). Regions of dyad symmetry were demonstrated in the 3' noncoding region that may be involved in regulation of transcription. Binz et al. (14) con-

cluded that botulinum neurotoxin type A was translated from a monocistronic RNA and that the mRNA did not also encode the hemagglutinin and other nontoxic proteins of the natural toxin complex. Thompson et al. (219) also concluded that a single open reading frame was translated, giving only the neurotoxin protein.

The sequence of the type A neurotoxin gene indicated that botulinum neurotoxin A does not possess a signal peptide in the terminal coding regions, supporting the notion that it is not a secreted protein. Cys residues are conserved at positions 1060 and 1280 of botulinum (and tetanus) toxins, and Cys-454 occurs at the same position in *C. botulinum* type A, B, and E toxins and in tetanus toxin (61). Cys-454 is the sole Cys residue in the N-terminal region of the H chain and is probably involved in disulfide bridging of the L and H chains. Cys-430 is also located at an identical position in botulinum and tetanus toxin L chains. Sequence analysis of botulinum type A toxin indicated that the H chain of type A neurotoxin had six histidine residues arranged in a motif which the authors suggested could be involved in the biological action of the toxin, possibly penetration through the nerve membrane. The deduced amino acid sequences of botulinum toxins had about 33% homology to tetanus toxin, and the H chains showed higher homologies than the L chains (14, 219). No homologies were detected to other proteins including ADP-ribosylating clostridial toxins.

The DNA sequences have also recently been obtained for other botulinum toxins including type D, C₁, and E neurotoxins and the C₂ ADP-ribosyltransferase (13, 71, 72, 87, 105, 106, 157). Highly homologous regions were detected among the various neurotoxin gene sequences and tetanus toxin gene. The C₂ gene was found to be unrelated to C₁ and D neurotoxin genes.

Structure and properties of nontoxic proteins of the toxin complex. Relatively little is known concerning the biochemistry and genetics of the nontoxic proteins associated with neurotoxin in toxin complexes. The type A complex contains at least two nontoxic proteins, one of which has hemagglutinating properties (115). Strains of *C. botulinum* that do not produce hemagglutinin have been isolated, and these form smaller complexes (12S and 16S) than are normally found (19S) (111, 135, 211). The in vitro addition of hemagglutinin to the 12S complex results in formation of a 19S complex with increased stability (111). Binding of the hemagglutinin was inhibited by a heat-stable, dialyzable substance that has not been isolated (211).

DasGupta (44) reported that the hemagglutinins of type A and B toxins were constructed through aggregation of two small units of about 15,000 and 20,000 M_r . Recently, Somers and DasGupta (204) studied nontoxic proteins from type A, B, C₁, and E toxin complexes. The proteins isolated from types A, B, and E had various degrees of hemagglutinating activity (Hn^+), while the protein from type E had no hemagglutinating activity (Hn^-). The type A Hn^+ and type B Hn^+ were serologically cross-reactive. Type A Hn^+ , type B Hn^+ , and type C Hn^+ were isolated as large aggregates (220,000 to 900,000 M_r), which were separated into multiple subunits of $\geq 17,000 M_r$ by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The type E Hn^- of 116,000 M_r did not aggregate. The sequences of the 10 to 33 amino-terminal regions of the 17,000, 21,500, 35,000, and 57,000 M_r subunits of type A Hn^+ and type B Hn^+ were determined. Each of the subunits had a unique sequence, indicating that the subunits were not homomers of smaller units. The subunits types A and B had remarkably similar

sequence identity; i.e., the 21,500 M_r subunits were identical and the 57,000 M_r subunits had 80% identity.

An understanding of the genetics of the hemagglutinin component of the toxin complexes is also developing. Oguma et al. (148) showed several years ago that the capacity to produce hemagglutinin in *C. botulinum* type C was transferred by phages either separately or together with toxin. Physical linkage of a hemagglutinin gene and toxin gene was confirmed, and it was shown that the toxin and hemagglutinin genes were transcribed in opposite directions. Tsuzuki et al. (224) cloned the gene encoding the main component of hemagglutinin produced by *C. botulinum* type C. The complete nucleotide sequence of the gene indicated that it encoded a protein of 33,000 M_r . At 62 bp downstream from the termination codon of the cloned 33,000 M_r subunit of type C Hn⁺ was an initiation codon followed by a coding sequence for at least 34 amino acids. Somers and DasGupta (204) found that the derived amino acid sequence of this open reading frame had 73 to 84% sequence identity with the 17,000 M_r subunits of type A Hn⁺ and type B Hn⁺ and significant similarity with the N terminus of type E Hn⁺. This observation raises the interesting possibility that genes for some of the subunits have a similar genetic arrangement and a common ancestral origin. It is interesting that a sequence homology has been proposed between tetanus toxin and the hemagglutinin of influenza virus (138), indicating a possible viral origin of the neurotoxin.

New findings in the mechanisms of action of the different types of botulinum toxins. Botulinum neurotoxins A to G are antigenically distinct yet have a number of structural and mechanistic similarities. All of the neurotoxin types cause a chemical denervation at the myoneural junction by inhibiting acetylcholine release. However, there appear to be subtle differences in the mechanisms of action of the neurotoxins. Toxin types A and E, type B, and type F apparently bind to distinct high-affinity acceptor regions with similar affinities (K_D 10^{-9} to 10^{-10} nM) in synaptosomes and at murine neuromuscular junctions (20, 45, 62, 110, 128, 228, 233). Binding may occur in regions composed of sialosyl residues and protein (184) and may first involve low-affinity association of the H chain followed by high-affinity attachment. In addition to binding to different acceptor regions on the nerve surface, botulinum toxin types A and B have been reported to affect neurotransmitter release differently (74, 137). Electrophysiological studies have shown that type A affects asynchronous neurotransmitter release, whereas type B does not (137). Furthermore, an increase in the intracellular Ca^{2+} concentration by ionophore treatment reverses inhibition by type A but not type B in synaptosome preparations (5), and aminopyridine more readily reverses type A than type B inhibition at the myoneural junction (74).

Both the H and L chains of the neurotoxin may be required for poisoning in invertebrate systems (123, 158). In mammalian peripheral motor nerve terminals, the L chain alone is active after it is internalized (15, 49). The precise mechanism of blockade by the L chain is unknown, but it must affect a general and important component of the secretory machinery in various classes of neurons. Botulinum toxin blocks the release of several classes of neurotransmitters at central and peripheral neurons (15, 120). Recently, it has been proposed that the L chain may act at an intracellular membranous or cytoskeletal site to inhibit neurotransmitter release (5, 120). Because of the extraordinary toxicity of botulinum toxin, it is likely that it has enzymatic activity and acts catalytically or triggers a cascade of events that decrease neurotransmitter release. The intracellular

substrate of botulinum toxin remains an elusive grail that is being pursued by several laboratories.

An objective in treatment of hyperactive muscles is to prevent possible systemic reactions which could result from spread of toxin through the blood. Antibodies could be used therapeutically by application to the injection site to help limit the diffusion of toxin and alleviate side effects such as ptosis (190), or it may also be possible to add the nontoxic H chain after toxin injection to block toxin binding to neighboring nerves. The most desirable approach to avoid spread would be to confine the paralyzing action within the presynaptic nerve. An interesting recent development is the finding that stabilized mRNA (3' polyadenylated and 5' capped) corresponding to the nucleotide sequence of tetanus toxin gene (L chain) injected into *Aplysia californica* cholinergic neurons in a bath depressed neurotransmitter release in less than 1 h (136). Similar results were found for mRNA of the L chain of botulinum A toxin, but only when the H chain was also added to the bath. The L chains of tetanus and botulinum neurotoxins were demonstrated to be synthesized in the presynaptic neurons, and onset of toxin action was slower than that of neurotoxins injected directly.

The subtle differences in botulinum toxin mechanisms among the various serotypes suggest that combinations of botulinum toxins could be more effective in clinical practice than any one type alone. Further work is needed to produce, stabilize, and test the clinical effectiveness of different types. Preliminary work indicates that types B (26) and F (187) are useful in controlling certain spasmodic muscle disorders.

Clinical use of pure neurotoxin compared with toxin complexes. Most recent information concerning the structure and pharmacology of botulinum toxin has been obtained with purified neurotoxins, but it is unlikely that these will be used in a clinical setting. The toxin complexes are much more stable than neurotoxins and can be diluted and formulated with retention of toxicity. Pure neurotoxins can be kept for several weeks to months in solution in the cold but are inactivated on dilution, formulation, and drying. No clinical trials on primates have been performed with purified neurotoxins.

Sellin et al. (195) reported that injection of 1 to 20 U of crystalline type A botulinum toxin into the lower hindlimb of the rat produced a paralysis that lasted for several days. In contrast, injection of more than 1,200 U of type B neurotoxin was required to produce paralysis. The duration of paralysis was compared after injection of 20 U of type A or 5,000 U of type B toxin. Type A toxin caused paralysis for up to 7 days after injection, whereas type B toxin caused paralysis for only 3 days and twitching became evident at 5 to 7 days. It was also reported (104, 194) that pure type A neurotoxin was much more effective than type E or F neurotoxin in eliciting lasting paralysis in the lower hindlimb of rats.

Tetanus Toxin

Tetanus toxin, like botulinum toxin, is produced by an anaerobic sporeforming rod that has a similar morphology to *C. botulinum* (86). Unlike botulinum toxin, tetanus toxin can enter into the central nervous system by retrograde intraxonal transport through motor nerves (17, 81, 132). It causes uncontrolled spasms of voluntary muscles by blocking the release of inhibitory transmitters including γ -aminobutyric acid and glycine (132). Tetanus toxin also has significant activity in decreasing acetylcholine release in cholinergic peripheral nerves when injected locally (54) and could possibly be used as an adjunct to or independently

from botulinum toxin for control of hyperactive muscles if the acquired immunity could be overridden. Tetanus toxin could also be used pharmacologically to transport substances to the central nervous system (17, 18). The biochemistry and pharmacological activities have been recently reviewed (17, 81). Here we consider aspects of the toxin that pertain to tetanus toxin production, stability, and potential use in medicine.

Crystalline structure. Tetanus toxin was originally purified by Pillemer et al. in the 1940s by precipitation methods. They obtained toxin crystals by carefully carrying out repeated precipitations in methanol and controlling the ionic concentration, pH, and toxin concentration (156). Tetanus toxin, like botulinum toxin, is a simple protein that does not contain lipid or carbohydrate (156, 165, 166). Unlike botulinum toxin, tetanus toxin does not occur complexed with protecting proteins and will not survive gastric passage or cause food poisoning in humans.

Although crystals of tetanus toxin were obtained in the 1940s by Pillemer et al. (156) from cold alcohol solutions, crystallization was not confirmed by others for several years. More recently, two-dimensional crystals of tetanus toxin have been isolated from ammonium sulfate solutions after incubation for several weeks at 4°C (40, 167). Robinson et al. (167) obtained two-dimensional arrays of native tetanus toxin formed at the interface between a solution of the toxin and a phospholipid monolayer containing ganglioside. Crystalline arrays were obtained only when all three components (toxin, phospholipid, and ganglioside) were present. The three-dimensional structure of tetanus toxin at 14-Å (1.4-nm) resolution appeared as an asymmetrical three-lobed structure that could interact with the phospholipid monolayer in two possible orientations (167). The analysis indicated that tetanus toxin is composed of differently shaped domains with different functions.

Biosynthesis and activation of tetanus toxin. Tetanus toxin is synthesized intracellularly as a single polypeptide of 150,000 *M_r* that is released from the cells on autolysis and is then modified by proteases present in the medium (81, 88, 89). The single-chain molecule is difficult to isolate (81), and proteolytic modifications of the toxin have caused considerable difficulties in the accurate characterization of the molecule. Single-chain toxin can be prepared from washed extracted bacterial cells (88, 162) and by inclusion of protease inhibitors and use of specific purification procedures (7, 165, 231). Purified preparations containing protease inhibitor can be stored for 4 to 6 weeks without proteolytic modifications and loss of toxicity (165).

Conversion of tetanus toxin to the nicked form increases toxicity (7, 81). *C. tetani* forms proteases that produce nicking in culture (231), but many other endoproteases will also activate the toxin (2). Three regions in the molecule are particularly susceptible to nicking (81, 129). Mild trypsin treatment of intracellular single-chain toxin yields two chains of about 95,000 and 50,000 *M_r*. The modified tetanus molecule is strongly held together by noncovalent bonds, and reduction of disulfide does not result in separation of the chains. Strong denaturants such as urea or SDS (81, 166) or purification techniques such as isoelectric focusing (2, 231) are required to dissociate the chains.

The H chain of tetanus toxin possesses a particularly susceptible region that can be cleaved with proteases such as trypsin or papain, yielding two fragments (B and C) (2, 89). The isolated H and L chains and fragments B and C are poorly toxic compared with intact tetanus toxin (2). Ahnert-Hilger et al. (2) reported that the nicking sites contributing to

toxicity are located within a region spanning no more than 17 amino acids, and the N and C termini are not altered during the modification. The separated chains were reconstituted to active toxin. By reconstitution experiments, the L chain was demonstrated to possess the paralyzing activity in isolated nerve-muscle preparations. The H chain is required for toxin entry into the nerve tissues and for axonal transport (2).

Large-scale production of tetanus toxin. Tetanus toxin is produced in deep culture by methods similar to those described for botulinum toxin. J. Howard Mueller, Pauline Miller, and associates at Harvard Medical School developed the methods currently used for production of tetanus toxoid. They experienced much frustration in obtaining consistent quality and the high titers of toxin required for toxoid demand (121, 142-144). They realized the importance of medium formulation in obtaining good-quality tetanus toxin, "If it were only possible to grow the tetanus organism on a medium containing only chemically defined substances of low molecular weight, it should become a relatively straightforward matter to study and control the factors involved in toxin production, and to obtain a uniform product free from any possible antigenic material other than the specific substance desired." (141). An extensive study was carried out to identify factors controlling tetanus toxin formation (143). On fractionation of components of the medium, the basis of good production was determined to be present in a pancreatic digest of casein. The key to good toxin production by *C. tetani* was later determined to be limitation of histidine (144). Abundant free histidine drastically decreased toxin production, while its limitation strongly increased titers (144). Since histidine is required for growth of *C. tetani*, it was necessary to find a method to limit the nutrient without stopping growth. Mueller and Miller found that providing histidine-containing peptides (e.g., glycyl-histidine) or histidine esters (e.g., acetyl-histidine) stimulated toxin production. Latham et al. (117) developed a protein-free medium which is currently used for tetanus toxin production. Mueller and Miller also isolated a high-producing strain (the Harvard or Massachusetts strain) (142) that is still widely used by many laboratories.

Tetanus toxin synthesis was found to be repressed by the addition of excess amino acids to the medium (223). Melanby (131) reported that glutamate addition to the Mueller and Miller growth medium decreased toxin formation but shortened the time necessary for autolysis. The results indicate that nitrogen nutrition controls toxin regulation in *C. tetani*. It is interesting that excess nitrogen also represses botulinum toxin synthesis in *C. botulinum* (119, 152). Careful adjustment of the levels of iron salts in the medium is also necessary for good tetanus toxin production (67, 142). The mechanisms of nutritional regulation and its importance in the biology of *C. tetani* and *C. botulinum* have not been further studied to our knowledge.

Tetanus toxin, like botulinum toxin, is produced in highest quantities by nonsporulating cultures (85, 145). Highly toxigenic cultures autolyzed thoroughly and did not form endospores during culture. During culture, tetanus toxin was present within the cell and was not released until cultures lysed (141, 162). As with *C. botulinum*, it appears that toxin formation is associated with autolytic growth and inversely associated with sporulation (145, 153). It would be of interest to determine whether specific transcription factors, e.g., sigma factors, regulate transcription of the toxin gene and whether these are preferentially expressed or activated in autolytic growth compared with sporulation.

Genetics of tetanus toxin. Tetanus toxin production has

been recognized as an unstable property for many years (68, 142). Attempts were made early to correlate the toxigenicity with the presence of bacteriophage. Phages were induced in *C. tetani* by treatment with mitomycin C, but induction did not affect toxin production (159, 160). Nontoxigenic mutants were readily isolated at high frequency (0.8 to 3.2%) from the Harvard strain A47 by treatment with various mutagenic agents including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, UV light, and rifampin (85). Hara et al. (85) found that cured, nontoxigenic strains still carried phages and proposed that plasmids could be involved in toxigenesis. Laird et al. (112) showed that toxigenicity was associated with the presence of a single large plasmid in 21 strains of diverse origin. Nontoxigenic derivatives were isolated, and each strain lost its plasmid. Two naturally occurring nontoxigenic strains were examined, and one was free of plasmids while the other contained a single large plasmid (112). The strains derived from the Harvard strain all contain a plasmid of 49 kb, which had identical restriction nuclease digestion patterns (112). By construction of a pool of nucleotide probes corresponding to the *N*-terminal amino acid sequence of tetanus toxin, Finn et al. (69) located the tetanus toxin gene to plasmid-related sequences. Surprisingly, strains with deletions in the plasmid still hybridized to the probes, suggesting that toxin gene sequences were still present but were not expressed to active product.

Eisel et al. (61) used a pool of oligonucleotides (heptadecamers) made up of all possible DNA sequences for *N*-terminal amino acids 8 to 13 of fragment C of tetanus toxin and screened plasmid preparations from toxigenic and nontoxigenic variants of the Harvard strain. Overlapping sequences that spanned the entire toxin gene were obtained from eight clones. The DNA fragments encoding tetanus toxin specified an open reading frame of 1,315 amino acids of 150,700 *M_r*. The open reading frame begins with an initiation codon for methionine, but purified toxin, like botulinum toxin, possesses proline at its *N* terminus and contains serine at the *N* terminus of the H chain (61). The molecular weights of the H and L chains calculated from the amino acid sequence are 98,300 and 52,288, respectively. Partial sequences reported for peptide fragments from the L chain (166) are only partly consistent with those obtained by nucleotide sequencing. The discrepancy may be caused by the extensive proteolytic processing that tetanus toxin undergoes following cell autolysis.

Computer searches using the primary sequence of tetanus toxin have not revealed primary structural similarities with any proteins other than botulinum toxin. Analysis of the primary sequence has also provided evidence that tetanus did not evolve by duplication of sequences within the H and L chains, which was earlier suggested by the similarities in amino acid compositions of the H and L chains (217) and by immunological similarities probed with monoclonal antibodies (227).

The availability of cloned tetanus gene fragments has enabled the production of tetanus toxin fragments for potential use as vaccines. Makoff et al. (124) expressed tetanus toxin fragment C in *E. coli* as 3 to 4% of the total cell protein. However, the coding sequence for fragment C is A+T rich and contains several codons rarely used in *E. coli*. Production was improved by replacing the coding sequence by a sequence optimized for codon usage in *E. coli* (125). More efficient translation of the mRNA was the most important factor for the increased expression. When the modified coding sequence was combined with improved promoter strength, fragment C was expressed as 11 to 14% of the cell

protein. Halpern et al. (83) cloned the sequence encoding fragment C and showed that the fragment expressed in *E. coli* retained ganglioside- and neuronal cell-binding activity. Recombinant fragment C was purified in one step by affinity chromatography. Recombinant fragment C was also immunogenic in mice and elicited antibodies that protected against tetanus toxin challenge. The availability of recombinant fragment C should be useful for a variety of research applications and for production of toxoid.

Pharmacological and medical applications of tetanus toxin. Tetanus acts primarily in the central nervous system and causes hyperactivity of the motor system and a spastic paralysis. Under specific conditions, tetanus toxin also inhibits peripheral neuromuscular transmission, resulting in a flaccid paralysis (82, 130). Tetanus toxin resembles botulinum neurotoxin in its structure and mode of action (81). Botulinum and tetanus neurotoxins have significant homology at the amino-terminal regions of the L and H chains, suggesting that at least portions of the respective genes evolved from a common ancestral gene.

Similarities in structure of tetanus and botulinum toxins have also been demonstrated in serological studies. Antibodies to type C botulinum toxin cross-reacted with other botulinum toxin serotypes and also reacted with tetanus toxin (149, 225). Tsuzuki et al. (225) found that a monoclonal antibody raised against botulinum toxin type E cross-reacted with botulinum toxin types B, C₁, and D and with tetanus toxin. Halpern et al. (84) developed antibodies against defined regions of the tetanus toxin to identify regions shared by tetanus and botulinum toxins. Synthetic peptides that corresponded to different regions of tetanus toxin were prepared and coupled to bovine serum albumin, which were used to immunize mice. Eleven of 13 peptides elicited antibodies that reacted with tetanus toxin in an enzyme-linked immunosorbent assay. Of 10 anti-tetanus peptide antibodies that reacted well with tetanus toxin, 1 reacted with botulinum toxin types B, C₁, and E but did not recognize type A. This antibody was made with a peptide corresponding to the amino-terminal end of the tetanus L chain, suggesting that this region is important in intoxication and that its structure is conserved in the two toxins. The antigenic region may be shielded in the native toxin but exposed on denaturation. Halpern et al. (84) also tested human tetanus immune globulin and mouse anti-tetanus serum for cross-reactivity with botulinum toxin, but none was detected. These results suggested that native forms of tetanus and botulinum toxins have little common surface antigenicity. This conclusion was also reached by Tsuzuki et al. (225), who prepared 306 monoclonal antibodies against the L chain of botulinum toxin type E and found that only 1 reacted with the other botulinum toxin types and with tetanus toxin.

Tetanus toxin has the unique ability to enter into the central nervous system through motor neurons. Because of the ability to travel up motor nerves, tetanus toxin or nontoxic fragments could provide a unique neurotropic agent to transport substances to the central nervous system (16-19). A 45-kDa nontoxic fragment, B-II_b (fragment C), that bound to toxin-binding sites on neuronal cell membranes and transported retrogradely from the axonal endings within the muscle to the motoneuronal perikaria was isolated (19). Bizzini et al. (18) constructed hybrid molecules consisting of the neurotropic fragment C and the I_{bc} fragment derived from tetanus toxin connected through disulfide linkage. The I_{bc} fragment was specifically carried to the central nervous system. Bizzini (17) also reported that fragment C

could compete with rabies virus for attachment to binding sites on neuronal cells and affected the rate of spread of rabies virus. Cloning and expression of high levels of fragment C should lead to further studies of targeted delivery to the central nervous system and possibly to control of virus infections.

Tetanus toxin also can act peripherally, causing a flaccid paralysis in the manner that characterizes botulinum toxin (54). H chains of both botulinum and tetanus toxins form channels in lipid bilayers (94). The H₂ fragment of the H chain of tetanus toxin was found to antagonize the action of botulinum toxin in phrenic nerve-hemidiaphragm preparations (201, 202). Tetanus toxin is about 2,000 times more toxic at central inhibitory nerves than at peripheral synapses (12, 54) and is about 1,000 times less toxic than botulinum toxin type A at the myoneural junction (82). Dreyer and Schmitt (55) proposed that tetanus toxin and botulinum toxin type A act at different sites in nerve inhibition of transmitter release. Botulinum toxins type B (74, 195), D (54), and F (104), but not A (74), appeared to act in a similar manner to tetanus toxin in affecting transmitter release from the myoneural junction. The combination of botulinum and tetanus toxins or the construction of chimeras could potentially be used to control neurological disorders.

MICROBIAL NEUROTOXINS THAT ALTER VOLTAGE-GATED SODIUM CHANNELS

Other microbial neurotoxins impair muscle activity in a way different from botulinum and tetanus toxins by their effect on the action potential at the sodium channel of a nerve axon. Saxitoxin and tetrodotoxin are two classical examples of microbial neurotoxins that block or close the passage of sodium ions through the channel. Toxins produced by other dinoflagellates also produce changes in the action potential at the sodium channel and are briefly described below.

Saxitoxin is a potent rapidly acting neurotoxin produced by the marine dinoflagellate *Gonyaulax catenella* (206) and some bacteria (102, 122). Like botulinum toxin, it was first observed as a food-borne toxin, causing food poisoning that occurred only at certain times from consumption of mussels, clams, and some other shellfish that are plankton feeders. Consumption of toxic shellfish results in symptoms described as numbness of the lips and fingertips within a few minutes followed by a progressive paralysis of the arms and legs along with the development of labored breathing and asphyxia. Death may occur within 2 to 24 h, depending upon the dose, from respiratory paralysis. After survival for 24 h the prognosis is good, and no lasting effects of the toxin have been observed. The oral dose that causes death from accidental consumption of toxic shellfish by humans is 1 to 4 mg (5,000 to 20,000 mouse units) depending upon the age and physical condition of the patient. A mouse unit (MU) is defined as the minimum amount needed to cause the death of an 18- to 22-g white mouse in 15 min, which is usually the maximum time in which death will occur (174, 205).

Saxitoxin was first purified and crystallized by Schantz et al. (172, 179), and its structure was determined by X-ray crystallography by Jon Clardy (175). Purified saxitoxin is a very hygroscopic water-soluble toxin and is described chemically as a tetrahydropurine base with pK_as at 8.5 and 11.5. It has a molecular weight of 299 as the free base. It has no UV absorption above 210 nm. As the dihydrochloride salt it is a white solid that is stable in acidic solution but loses activity above pH 7. The paralyzing action of saxitoxin or its

binding at the receptor of the sodium channel depends upon the presence of a hydrated ketone group in a particular position in the molecule. Reduction of this group to the alcohol results in the loss of over 99% of the binding and paralyzing activity.

The neurotoxic action of saxitoxin is due to its specific binding, even at extremely low concentrations (10⁻⁹ M), at the sodium channel of excitable membranes and preventing the passage of sodium ions through the sodium channel, thus blocking an impulse. The action or binding is concentration dependent, and binding is reversible. Controlled application of the toxin has been suggested as a possible local anesthetic. The effective dose in animals is relatively close to the lethal dose, as indicated by the steepness of the response curve, and pharmaceutical companies have not pursued its use in humans. However, when saxitoxin is mixed in small amounts with many classes of anesthetics, the effectiveness of the anesthetic action is greatly extended (1). The addition of 1 µg of saxitoxin to a 1% solution of a typical anesthetic (1 part in 10,000) such as procaine increased the time of effective action two- to threefold (1). The result is not an additive one (1). The addition of saxitoxin to procaine as well as to other anesthetic compounds will also reduce the dose required to obtain a desired effect. The reason for this unusual action of saxitoxin with anesthetic compounds has not been fully explained, but the molecule must play an important part in nervous function in the presence of other substances that act on the nervous system. Saxitoxin and tetrodotoxin have been important in the establishment and characterization of the sodium channel in myelinated and unmyelinated nerve membranes (90, 93, 102, 163, 164) and for the study of related diseases such as multiple sclerosis.

Another species of dinoflagellates, *Gonyaulax tamarens* var. *excavata*, produces saxitoxin substituted with sulfate and sulfonic acid groups (63, 102). These substituted toxins have a lower specific toxicity or binding at the sodium channel than saxitoxin does, but they should not be overlooked for possible medical use.

Tetrodotoxin was originally found in the roe, ovaries, and liver of the puffer or globe fish (*Tetraodontidae*) caught in the western Pacific ocean and was at first believed to be exclusively produced by this fish. More recently it has also been found in various other animals including the California newt, octopus, and frog (234) and in marine bacteria (51, 199, 220, 235). Dinoflagellates have been proposed as the original source of the toxin in puffer fish, which acquire it through the food chain (234).

The action of tetrodotoxin is like that of saxitoxin in blocking the sodium channel of excitable membranes of nerve and muscle tissue. In fact, it has been shown that both tetrodotoxin and saxitoxin block the inward current of sodium ions at equally low concentrations of 10⁻⁷ to 10⁻⁹ M and occupy the same receptor sites at the sodium channel (63, 103). The basic structure of tetrodotoxin is markedly different from that of saxitoxin and is chemically described as aminoperhydroquanzoline, with a molecular weight of 319. Although the two toxins are basically different in structure, they may be similarly classified as heterocyclic guanidines because of the guanidium group common to each toxin. Kao and Nishiyama (103) first proposed that the guanidinium moieties of each toxin might enter at the sodium channel like guanidine and that the bulk of the remaining part of the molecule prevented the passage of the sodium ion.

Although this hypothesis may be consistent with many aspects of the action of the toxins, it appears that the chemical makeup of the molecule as well as the guanidine

group is involved. The reduction of the hydrated ketone group to an alcohol in the saxitoxin molecule completely destroys its effectiveness as a blocker of the sodium channel, and changes in the structure of tetrodotoxin also affect its bonding (102). The purpose here is to point out in a general way the nature of the two toxins and how they might affect the action of other toxins used for treatment of hyperactive muscles. The fact that saxitoxin enhances the action of local anesthetics has raised some thoughts on the relation of one toxin to another on an excitable membrane. Reviews by Catterall (36), Kao et al. (102, 103), and Borison et al. (22) give detailed descriptions of the action of the microbial neurotoxins saxitoxin and tetrodotoxin (binding site 1 at the sodium channel) and compare them with neurotoxins from other nonmicrobial sources that affect the sodium channel, such as veratridine, aconitine, batrachotoxin, grayanotoxin, and the low-molecular-weight basic polypeptide toxins isolated from scorpion venoms, fish-hunting cone snails, and sea anemone nematocysts (binding site 2 at the sodium channel). These reviews point out the various mechanisms by which toxins might affect the nervous system via action at the sodium channel. From the proposed action it seems reasonable to assume that there may be value in the use of combined toxins for control of nervous activity.

It is quite interesting that the action potential at the sodium channel is also affected by certain substances, such as guanidine and 3,4-diaminopurine, that reverse or bypass the blocking action of botulinum toxin. Although these substances are not particularly good antidotes for the toxin, their action indicates a relationship between the action potential at the sodium channel and the liberation of a neurotransmitter at the nerve ending. One might assume, therefore, that substances such as saxitoxin, tetrodotoxin, or others that alter the action potential at the sodium channel should warrant further investigation for possible medical application. Guanidine, an effective substitute for sodium in action potential generation in excitable membranes (93), is also reported to relieve symptoms of botulism (39), suggesting that there may be interactions of toxins at the myoneural junction, a field that warrants further study.

Besides the microbial neurotoxins described thus far, there are other, less well understood microbial neurotoxins that may be found valuable for nerve and muscle control mainly because of their action at the sodium channel. *Gymnodinium breve*, a marine dinoflagellate responsible for the Florida red tides and the tremendous fish kills in that area, produces several toxins, two of which are neurotoxins designated brevitoxins A and B, that have some action at the sodium channel (22). These two toxins are lipid-soluble polyethers with a molecular weight around 900. Brevetoxin A has an indirect action on the sodium channel in that it enhances channel activity in the presence of toxins that bind to receptor site 2 at the sodium channel, but not to receptor site 1.

Gambierdiscus toxicus, a tropical reef-dwelling dinoflagellate, produces several toxins, including one designated ciguatera toxin, which opens voltage-dependent sodium channels in cell membranes (186). This toxin is a lipid-soluble polyether with a molecular weight of 1112 and is concentrated as it is passed up the food chain to large predatory reef fish consumed by humans. The disease in humans affects both gastrointestinal and neurological systems. The neurological symptoms usually begin within 24 h and may last a month or more, indicating nerve blockage or damage requiring regeneration of nervous tissue. Afflicted persons experience cir-

cumoral paresthesias, paresthesias or paralysis of the extremities, and muscle pain.

Natural blooms of the freshwater blue-green alga (cyanobacterium) *Aphanizomenon flos-aquae* which occur periodically in lakes of the northern United States and certain provinces of Canada have caused poisoning of farm animals from drinking the water. This organism produces several toxins including saxitoxin and neosaxitoxin. Another species of this group, *Anabena flos-aquae*, produces a substance that affects acetylcholine receptors in muscle membranes (35, 97). Some mycotoxins affect the nervous system in various ways. Slaframine, upon biological conversion to a quaternary amine, causes excessive salivation in farm animals and acts similarly to acetylcholine (30, 41). These organisms and other microorganisms produce other neural toxins, but little is known of their action and importance in pharmacology and physiology.

CONCLUSIONS

Botulinum toxin type A has been found useful for the treatment of many hyperactive muscle disorders by intramuscular injection, and the FDA has licensed the toxin for treatment of strabismus, blepharospasm, and hemifacial spasm. It is the first microbial toxin to be used for human treatment. Because it is injected into humans, purity is of prime importance and, therefore, during the production by culturing and purification, it must not be exposed to any substances that might be carried in trace amounts to the crystalline toxin and cause undue reactions in the patient. Injection of the toxin into muscle tissue has opened a new field of investigation into the action of the toxin on muscle and nerve tissue and has been beneficial to many humans who suffer from dystonias.

Types of botulinum toxin other than type A toxin and perhaps tetanus toxin may be useful for human treatment if patients develop immunity to type A toxin. Saxitoxin stimulates and prolongs the action of local anesthetics, suggesting the use of combined toxins for human treatment. Some microbiological toxins are described for possible use alone or combined with botulinum toxin for medical treatment.

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Botulinum Neurotoxins Are Zinc Proteins*

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The available amino acid sequences of 150-kDa botulinum and tetanus neurotoxins show the presence of a closely homologous segment in the middle of the light chain (NH₂-terminal 50 kDa), which is the intracellularly active portion of the toxin. This segment contains the zinc binding motif of metalloendopeptidases, HEXXH. Atomic adsorption analysis of botulinum neurotoxins (serotypes A, B, and E) made on the basis of this observation demonstrated the presence of one zinc atom/molecule of 150-kDa neurotoxin. Conditions were found for the removal of the zinc ion with chelating agents and for the restoration of the normal metal content. The conserved segment, which includes the zinc binding motif, was synthesized and shown to bind [⁶⁵Zn]²⁺.

Chemical modification experiments indicated that two histidines and no cysteines are involved in Zn²⁺ coordination in agreement with a probable catalytic role for the zinc ion. The present findings suggest the possibility that botulinum neurotoxins are zinc proteases.

Botulinum neurotoxins (BoNT)¹ are produced in seven different serotypes (A, B, C1, D, E, F, and G) by *Clostridium botulinum* and by other species of the same genus (Simpson, 1989; Hatheway, 1990). They are the most potent bacterial protein toxins. All the clinical symptoms of botulism, including the flaccid paralysis, are due to BoNT, which blocks acetylcholine release at the neuromuscular junction. Their enormous potency has been attributed to their neurospecificity and to a yet unknown intracellular enzymic activity as it is the case for all bacterial protein toxins with cytosolic targets (Simpson, 1989; Alouf and Freer, 1991).

BoNTs are produced as 150-kDa single polypeptide chains. Proteolytic cleavage within a narrow region generates a two-chain form of BoNT composed of an H chain (100 kDa) and an L chain (50 kDa) held together by a disulfide bridge and noncovalent bonds (Fig. 1). The two-chain form is generally more active than the single chain (DasGupta, 1989). The H chain is responsible for the neurospecific binding of BoNT to

peripheral nerve cells and presumably also for the cytosolic translocation of the L chain (Montecucco, 1986; Niemann, 1991). The L chain is the intracellularly active portion of the neurotoxin that blocks neuroexocytosis (Poulain *et al.*, 1988, 1990, 1991). However, despite all efforts, neither its intracellular mode of action nor its target is yet known.

We suggest here the possibility that BoNTs are metalloendopeptidases. This suggestion is based on the following findings. All clostridial neurotoxins, whose sequences are available, contain the zinc binding motif of zinc endopeptidases. Consequently, we investigated the possibility that BoNTs are metalloproteins by measuring the metal content of serotypes A, B, and E. These three serotypes were chosen because they are most frequently involved in human botulism (Hatheway, 1990) and are available as highly pure preparations. We found that indeed one atom of zinc is bound per molecule of BoNT, this binding is reversible, and histidines are involved in zinc coordination, as in all zinc endopeptidases.

MATERIALS AND METHODS

Purification of Neurotoxins and Related Fragments

BoNT serotypes A, B, and E were produced and purified as previously described (DasGupta and Rasmussen, 1983; DasGupta and Sathymoorthy, 1984; DasGupta and Woody, 1984). Serotypes A and E were in two-chain and single chain forms, whereas serotype B was essentially single chain. BoNT/B and BoNT/E were nicked with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Serva) as described by Sathymoorthy and DasGupta (1985), and the cleavage was blocked by adding a 4-fold excess of soybean trypsin inhibitor.

The H and L chains of serotype A were separated and purified as reported previously (Sathymoorthy and DasGupta, 1985). The 50-kDa carboxyl-terminal half of the H chain (H_C) and BoNT serotype A and the remaining 100-kDa fragment (L-H_N, the L chain, and the 50-kDa NH₂-terminal half of the H chain) were isolated following a procedure developed by Gimenez and DasGupta.² 5.0 mg of BoNT serotype A diluted to 0.5 mg/ml in 50 mM ammonium acetate, pH 4.1, was digested with pepsin (1:30, w/w) for 30 min at 35 °C. The reaction was blocked by adding 2 M Tris chloride, pH 8.8 (final concentration, 100 mM), and 1 µg/ml pepstatin. The reaction mixture was dialyzed extensively against 20 mM sodium phosphate, pH 8.0, and applied to a DEAE-Sephadex A-50 column (1.5 × 6.0 cm) equilibrated in the same buffer. H_C was recovered in the void volume, and the L-H_N fragment was eluted with a linear gradient of increasing sodium chloride; both were precipitated by ammonium sulfate (39 g/100 ml). Concentrations of H_C and L-H_N were determined from the absorbance at 278 nm using extinction coefficients of 1.7 and 1.27 M⁻¹ cm⁻¹, respectively.

Diphtheria toxin (DT) was prepared as described (Rappuoli *et al.*, 1983).

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¹ The abbreviations used are: BoNT, botulinum neurotoxin; DEPC, diethyl pyrocarbonate; DT, diphtheria toxin.

² A. Gimenez and B. R. DasGupta, manuscript in preparation.

Determination of Metal Content

Each material (glassware, dialysis bags, etc.) used in metal determination experiments was previously rinsed with Milli-Q grade water (conductivity > 10 megaohms). Buffers were prepared with chemicals of the highest purity available with respect to the presence of heavy metals and pretreated with Amberlite MB-3 (Sigma). Before metal determination, the neurotoxins and the fragments thereof were extensively dialyzed at 4 °C against 150 mM Tris chloride, pH 7.4, or 10 mM HEPES-Na, 100 mM sodium chloride, pH 7.0. Protein samples and dialysis buffer were analyzed for zinc, cobalt, copper, iron, manganese, and nickel with a Perkin-Elmer 4000 atomic absorption flame spectrophotometer with impact bed loading after standardization for each ion in the linear range of concentration (0–0.5 ppm for zinc, nickel, and cobalt; 0–1 ppm for copper; 0–5 ppm for iron and manganese).

Zinc Removal and Reuptake

BoNT serotypes A, B, and E were diluted to 0.5–1.0 mg/ml with 150 mM Tris chloride, pH 7.4, and incubated in the presence of 10 mM Na-EDTA for 60 min at 37 °C. Samples were dialyzed extensively against the same buffer without EDTA at 4 °C before zinc content was determined. Zinc reuptake was accomplished by adding 100 μ M zinc chloride dissolved in 150 mM Tris chloride, pH 7.4, to Zn^{2+} -depleted BoNT. After 60 min at 37 °C, the samples were extensively dialyzed against the same buffer without Zn^{2+} at 4 °C, and metal content was determined.

Diethyl Pyrocarbonate Modification

Native and zinc-depleted BoNT serotypes A, B, and E, between 1.5 and 2.5 μ M in 50 mM sodium phosphate, pH 7.8, after filtration through a 0.22- μ m filter (Anotec, Oxford, United Kingdom), were treated with DEPC in three consecutive additions of a 35-fold molar excess of reagent with respect to the toxin. DEPC was taken from a freshly prepared solution in absolute ethanol. The reaction was carried out at 25 °C and was monitored by simultaneous recording of the differential absorbances at 243 and 278 nm as previously described (Miles, 1977; Papini *et al.*, 1989) in a Perkin-Elmer Lambda 5 spectrophotometer. Modifications of histidine and tyrosine residues were estimated based on a differential extinction coefficient of 3,200 $\text{M}^{-1}\text{cm}^{-1}$ at 243 nm for *N*-carbethoxyhistidine and of $-1,310\text{ M}^{-1}\text{cm}^{-1}$ at 278 nm for *O*-carbethoxytyrosine.

Titration of Free Thiol Groups

Native or zinc-depleted BoNT serotypes A, B, and E, 1 μ M in de-aerated 50 mM sodium phosphate buffer, pH 7.8, were incubated with 0.4 mM 5,5'-dithiobis-(2-nitrobenzoic acid) at 25 °C. The absorbance at 410 nm was determined against a blank without neurotoxin. Based on a molar absorptivity of 13,600 $\text{M}^{-1}\text{cm}^{-1}$ at 412 nm for the 2-nitro-5-thiobenzoate anion, sulfhydryl content was calculated as moles of free thiol groups/mol of BoNT (Ellman, 1959; Schiavo *et al.*, 1990).

Peptide Synthesis

The segment of BoNT serotype B, which spans 15 residues (226–240, $\text{H}_2\text{N-Ile-Leu-Met-His-Glu-Leu-Ile-His-Val-Leu-His-Gly-Leu-Tyr-Gly-COOH}$) was prepared by solid phase synthesis with a SMPS 350 automatic synthesizer (Zymsser Analytic, Frankfurt, Germany) employing Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry. The product was detached from the resin with 93% trifluoroacetic acid purified by high pressure liquid chromatography on a Vydac C18 column (Vydac, CA), and its amino acid sequence was verified by automatic Edman degradation on an Applied Biosystems microsequencer (model 475A).

Determination of [^{65}Zn] $^{2+}$ Binding

Zinc Overlay—0.6–20 pmol of BoNT serotypes A and E in their native and zinc-depleted forms, before and after DEPC treatment (as mentioned above), as well as 20–600 pmol of the synthetic peptide, were dot blotted onto nitrocellulose paper stripes (porosity, 0.22 μ m) (Hoefer, CA). The same amount of diphtheria toxin was dot blotted as the control. The stripes were rinsed for 5 min in 25 mM Tris chloride, pH 7.5, containing 100 mM sodium chloride and then incubated for 60 min at room temperature in the presence of 50 nM [^{65}Zn] Cl_2 (Amersham, U.K.) (specific activity, 575 mCi/mg zinc) in the same buffer. Unbound [^{65}Zn] Cl_2 was removed by washing the stripes six times with 25 mM Tris chloride, 100 mM sodium chloride, pH 7.5.

The amount of bound [^{65}Zn] $^{2+}$ was determined by exposing the dried stripes to Kodak X-Omat films at $-80\text{ }^\circ\text{C}$.

Gel Filtration Chromatography—0.5 nmol of BoNT serotype E or 1.2 nmol of DT were incubated with 1.15 μ Ci of [^{65}Zn] Cl_2 in 150 μ l of 100 mM Tris chloride, 50 mM sodium chloride, pH 7.0, for 60 min at 25 °C and then were applied onto a Sephadex G-25 medium column (Pharmacia LKB Biotechnology Inc.) (100 \times 8 mm), equilibrated, and eluted with the same buffer. Void volume (4.0 ml) was determined with blue dextran (Pharmacia). 0.9-ml fractions were collected, protein elution was monitored by UV absorption at 280 nm, and radioactivity was measured by counting in a Packard Multi-Prias Gamma counter. 0.5 μ Ci of [^{65}Zn] $^{2+}$ coeluted with the protein peak of BoNT, whereas background amounts were found in the DT peak.

Flow Dialysis

Binding of [^{65}Zn] $^{2+}$ to the BoNTs was measured by the flow dialysis technique of Colowick and Womack (1969). The apparatus consisted of two cylindrical chambers (10 mm diameter; volumes: upper chamber, 1 ml; lower chamber, 0.18 ml) separated by a dialysis membrane. The toxins were preincubated for 45 h at 4 °C with 0.3 μ M [^{65}Zn] $^{2+}$. The upper chamber routinely contained 1.7–2.7 μ M BoNT and 0.3 μ M [^{65}Zn] $^{2+}$ in a 400- μ l volume of 20 mM Tris-Cl, 120 mM NaCl, 0.1 mM CaCl_2 , 5 mM MgCl_2 , pH 7.45, at room temperature. The lower chamber was perfused (0.5 ml/min) with the same buffer without zinc, and 0.5-ml fractions were collected and counted in a Packard Cobra Autogamma 5003 counter. 1–2- μ l aliquots of ZnCl_2 were added to the upper chamber every 6 fractions.

Toxicity Tests

The neuromuscular activity of BoNTs was tested by intravenous injection into BALB/c mice as described by Boroff and Fleck (1966).

RESULTS AND DISCUSSION

Sequence Comparison—The cDNA-derived amino acid sequences of BoNT serotypes A, B, C1, D, and E and from *Clostridium butyricum* as well as that of tetanus neurotoxin are known (Eisel *et al.*, 1986; Fairweather and Lyness, 1986; Hauser *et al.*, 1990; Binz *et al.*, 1990a, 1990b; Niemann, 1991; Thompson *et al.*, 1990; Poulet *et al.*, 1992). They show an overall low degree of homology with a few segments of close similarity. The most conserved segment among these clostridial neurotoxins, located in the central part of the light chain, is reported in the lower part of Fig. 1. The figure also shows that this segment includes the zinc binding motif of zinc endopeptidases (Vallee and Auld, 1990a).

Metal Content of Botulinum Neurotoxins—The above observation suggested to us the possibility that also the BoNTs are zinc proteins. To determine the nature and amount of metals bound to BoNT, highly purified preparations of BoNT serotypes A, B, and E, each in the two-chain form (their SDS-polyacrylamide gel electrophoresis profiles are in Fig. 2A), were subjected to atomic adsorption analysis. Fig. 2B shows that all three BoNT serotypes contain approximately 1 atom of zinc/molecule of 150-kDa toxin. Nicking of the BoNT serotypes B and E did not modify significantly the metal content (not shown). Analysis of the two peptic fragments of BoNT serotype A showed that the zinc atom is bound to the 100-kDa L- H_N fragment. The zinc content of the 50-kDa carboxyl-terminal fragment H_C was below detection limit. It was not possible to determine the metal content of the isolated 50-kDa L chains because separation of L and H chains required urea and dithiothreitol (Sathyamoorthy and DasGupta, 1985), which released the metal from the protein (not shown). Cobalt, copper, iron, manganese, and nickel were measured and found to be below detection limits.

Results presented in Fig. 2B also show that the Zn^{2+} atom was removed by treatment with EDTA. The loss of Zn^{2+} caused by EDTA was reversible because the zinc ion was reacquired by BoNT upon incubation in a Zn^{2+} -containing medium. On the contrary, Zn^{2+} reuptake by purified L chain

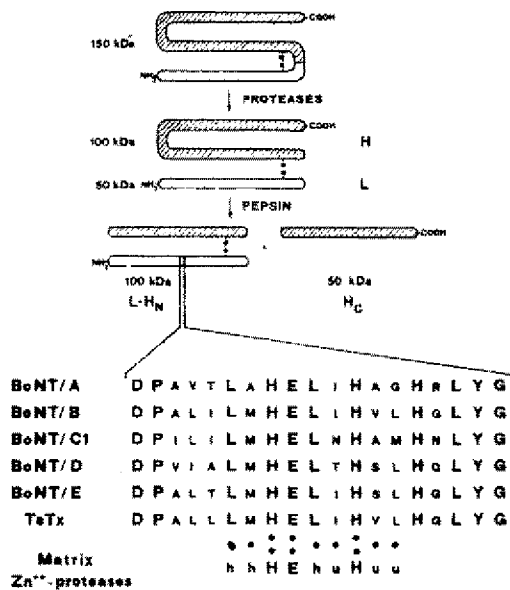


FIG. 1. Schematic structure of clostridial neurotoxins and sequence comparison of their histidine-rich segment with the zinc binding motif of metalloendopeptidases. Clostridial neurotoxins are produced as a single 150-kDa chain which is later cleaved by proteases at an exposed loop to generate the active two-chain toxin. The H chain (H) can be further cleaved with pepsin generating the 50-kDa H_C fragment. The most conserved segment of the sequences of BoNT A, B, C, D, and E and of tetanus toxin (TeTx), located in the central part of the L chain (L), is aligned, and it is shown to contain the Zn²⁺ binding motif of matrix metalloproteases; *h*, hydrophobic; *u*, uncharged. Double dots indicate residue conservation and single dots indicate conservation of residue properties.

of BoNT serotype A (the only L chain we have tested) was very low (not shown). This difference with the parent 150-kDa molecule indicates a partial alteration of the Zn²⁺ binding site of the L chain probably due to exposure to dithiothreitol and urea during its separation from H chain and isolation.

Zn²⁺ was also removed, with lower efficiency, by diethylenetriamine pentaacetic acid, a chelating agent specific for heavy metals (Arslan *et al.*, 1985). *O*-Phenantroline and dipicolinic acid, which are frequently used as heavy metal complexing agents, caused protein aggregation and an irreversible loss of zinc.

Zinc Coordination—The three-dimensional structures of three zinc metalloendopeptidases, thermolysin (Matthews *et al.*, 1972), *Bacillus cereus* neutral protease (Paupit *et al.*, 1988), and *Pseudomonas aeruginosa* elastase (Thayer *et al.*, 1991), have been resolved by x-ray crystallography. In these enzymes, the Zn²⁺ atom is bound via a tetrahedral coordination with the two histidines of the motif HEXXH, while the glutamic residue binds a water molecule, which is the third Zn²⁺ ligand. The fourth ligand is a glutamic residue that has been identified in bacterial zinc metalloendopeptidase but whose position is still to be determined in matrix metalloendopeptidases (Vallee and Auld, 1990a, 1990b) and in the clostridial neurotoxins.

The role of histidines in Zn²⁺ binding in the botulinum neurotoxin was tested with chemical modification experiments. DEPC is a protein-modifying agent specific for histidines, which are converted to *N*-carboxyhistidines with a characteristic absorbance at 243 nm (Miles, 1977). Figure 3 shows that a different number of histidine residues was modified per molecule of the three toxins. This is related to the different histidine contents of the three BoNTs (13, 7, and 14 for serotypes A, B, and E, respectively) and to their accessi-

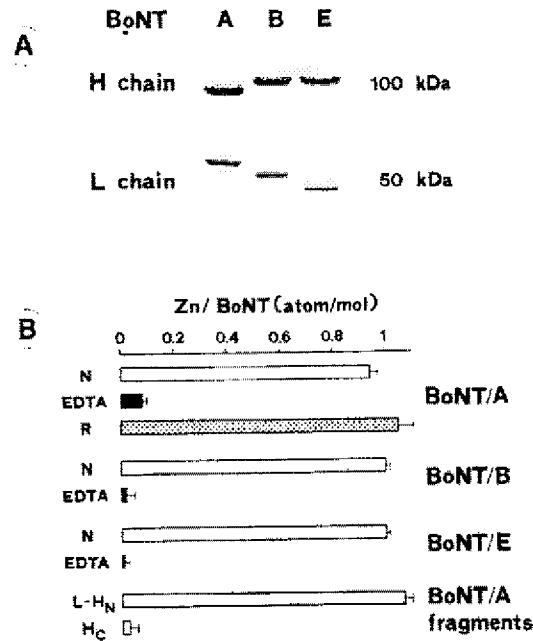


FIG. 2. Zinc content of botulinum neurotoxins A, B, and E. A, SDS-polyacrylamide gel electrophoresis and Coomassie Blue-stained samples of BoNT serotype A (lane A), B (lane B), and E (lane E). B, amounts of zinc bound to the dichain form of BoNT serotypes and to the two peptic fragments of BoNT/A, 100-kDa L-H_N and 50-kDa H_C, before or after EDTA treatment, measured by atomic adsorption (details under "Materials and Methods"). N, native BoNT; EDTA, BoNT treated with EDTA and dialyzed; R, BoNT depleted of zinc with EDTA, incubated with 100 μM ZnCl₂, and dialyzed. Bars are \pm S.D. of three different measurements.

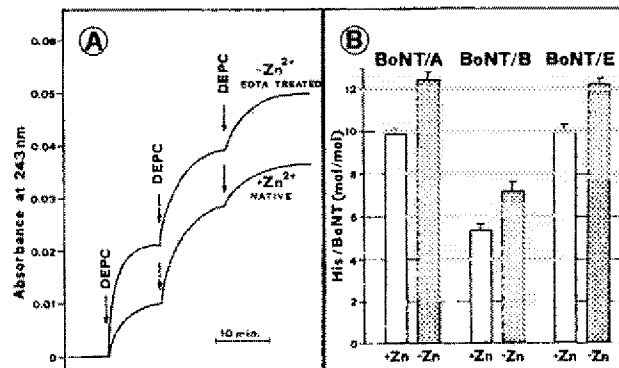


FIG. 3. Histidine titration with DEPC of botulinum neurotoxin serotypes A, B, and E before and after Zn²⁺ depletion. A, the increase of absorbance at 243 nm indicates the progressive formation of *N*-carboxyhistidine; successive addition of DEPC, necessary because of rapid DEPC decomposition in water, is indicated by arrows. B, amount of DEPC-modified histidines in the three BoNTs tested here before (empty bars) and after (dotted bars) Zn²⁺ removal. Bars are \pm S.D. of at least three different experiments.

bility to DEPC. However, for each neurotoxin, two additional histidines/BoNT molecule were modified when they were in their Zn²⁺-depleted form. Parallely, there was no modification of tyrosines as monitored at 278 nm.

This result indicates that (i) the Zn²⁺ ion of BoNT protects two histidine residues from DEPC modification and (ii) these two histidines become accessible to DEPC when the zinc atom is absent. Each of the three BoNTs was unable to regain the Zn²⁺ atom after Zn²⁺ depletion and DEPC modification (see

below). It was not possible to test the effect of Zn^{2+} depletion on the neuroparalytic activity of BoNT because of the reversibility of Zn^{2+} depletion and because Zn^{2+} -undepleted and DEPC-modified BoNT is no longer toxic (not shown).

Cysteine residues coordinate Zn^{2+} in several proteins in which the zinc atom plays a structural role, such as in aspartate carbamoyltransferase, zinc finger proteins, and metallothioneins (Vallee and Auld, 1990a, 1990b). Titration of the sulfhydryl group of BoNT serotypes A, B, and E before and after Zn^{2+} depletion gave the same value (not shown), thus indicating that cysteines are not implicated in zinc binding in BoNTs.

These results are in agreement with the prediction, based on the sequence comparison of Fig. 1, that histidines, and not cysteines, are involved in Zn^{2+} coordination. Moreover, they suggest that the zinc ion of BoNT plays a catalytic role as in all zinc enzymes with histidine coordination (Vallee and Auld, 1990a, 1990b).

[$^{65}Zn^{2+}$ Binding to BoNT and the Synthesized Peptide, Residues 226–240]—To gain further evidence that the central segment of the BoNTs L chain is responsible for Zn^{2+} coordination, the peptide $H_2N-Ile^{226}-Leu-Met-His-Glu-Leu-Ile-His-Val-Leu-His-Gly-Leu-Tyr-Gly^{240}-COOH$, spanning the corresponding sequence of BoNT/B, which includes the putative zinc binding motif, was synthesized. Results of dot blot experiments (Fig. 4) show that this peptide binds [$^{65}Zn^{2+}$] and suggest that the conserved histidine-rich segment of BoNTs and tetanus toxin can take part in zinc coordination. Fig. 4 also shows that both the zinc-depleted ($-Zn^{2+}$) and the native BoNT ($+Zn^{2+}$) serotypes A and E bind [$^{65}Zn^{2+}$]. This indicates that bound zinc is exchangeable. This exchange is not a peculiarity of the dot blot assay because it also occurred with BoNT in solution (Fig. 5).

BoNTs depleted of zinc and subsequently treated with DEPC were unable to bind [$^{65}Zn^{2+}$] (Fig. 4), whereas a parallel DEPC treatment of the native BoNT did not affect its ability

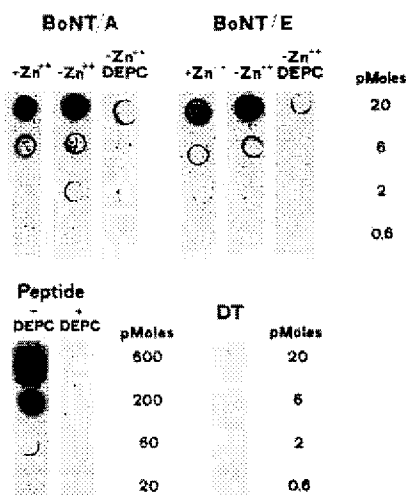


FIG. 4. [$^{65}Zn^{2+}$] binding to botulinum neurotoxins A and E and to the histidine-rich conserved peptide 226–240 of BoNT/B. Protein (0.6–20 pmol) or peptide (20–600 pmol) samples, blotted onto nitrocellulose paper, were incubated in 50 nM [$^{65}ZnCl_2$]. After washings, the paper strips were dried and autoradiographed. Samples are as follows: $+Zn^{2+}$, native BoNT; $-Zn^{2+}$, BoNT treated with EDTA and dialyzed; $-Zn^{2+}$ DEPC, BoNT treated with EDTA, dialyzed, and treated with DEPC; peptide, peptide 226–240 of BoNT/B not treated (–) or treated (+) with DEPC (details under “Materials and Methods”). Native BoNT treated with DEPC binds [$^{65}Zn^{2+}$] as the untreated control ($+Zn^{2+}$) (not shown). DT refers to samples of diphtheria toxin.

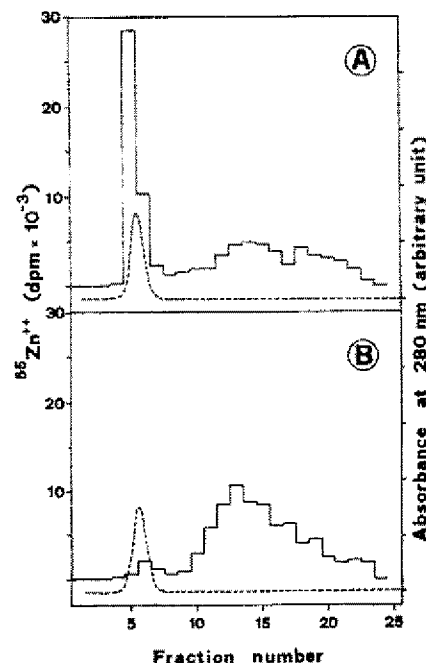


FIG. 5. Zinc exchange on botulinum neurotoxin serotype E. Elution of BoNT/E or diphtheria toxin from a Sephadex G-25 column after incubation of the toxins with 200 pM [$^{65}ZnCl_2$] for 60 min at 25 °C. The broken lines show absorbance at 280 nm, and the continuous lines report the amount of radioactivity associated to the various fractions. A, BoNT serotype E; B, diphtheria toxin. Under the present conditions, 45% of added [$^{65}Zn^{2+}$] coeluted with the BoNT peak, and practically none coeluted with the diphtheria toxin peak.

TABLE I
Flow dialysis and Scatchard analysis for [$^{65}Zn^{2+}$] binding to BoNT/A, B, and E

Toxin	K_D nM	n	K_D μM	n
BoNT/A ^a	60–80	1–1.1	1.1–1.4	2.2–3.1
BoNT/B ^b	90–100	0.7–1.1	1.6–2.2	1.9–2.6
BoNT/E ^b	80–130	1	1.4–2.4	2.5–3.0

^a Range of values found in three different experiments.

^b Range of values found in two different experiments.

to exchange the Zn^{2+} atom (not shown). This result indicates that histidines are involved in zinc coordination in BoNT. The dot blot assay used here appears to be very specific since diphtheria toxin, which has three histidines interspaced by three residues ($His^{484}-X-X-X-His-X-X-X-His^{492}$), does not show any sign of [$^{65}Zn^{2+}$] binding (Figs. 4 and 5).

Affinity of Zinc Binding to Botulinum Neurotoxins—The affinity of zinc binding to the three BoNTs was assayed by equilibrium dialysis, employing [$^{65}Zn^{2+}$] as tracer (Colowick and Womack, 1969; Papini *et al.*, 1989). Table I shows that the three BoNT serotypes bind zinc very similarly. The three BoNTs have a single high affinity binding site (n close to 1) with comparable K_D values and also show multiple lower affinity sites that are not occupied in the purified toxins to account for the above described atomic adsorption data.

Conclusions—The present paper demonstrates that botulinum neurotoxins (serotypes A, B, and E) contain one atom of zinc/molecule of 150-kDa protein and that the Zn^{2+} atom can be reversibly removed with EDTA. This work also shows that histidines, and not cysteines, are involved in metal coordination, thus suggesting that the zinc atom plays a catalytic rather than a structural role (Vallee and Auld, 1990a, 1990b).

Comparison of amino acid sequences and [^{65}Zn] $^{2+}$ binding experiments suggests that the histidine-rich segment, conserved among clostridial neurotoxins, is involved in zinc coordination. This segment contains the zinc binding motif of metalloendopeptidases, including the glutamic residue directly involved in catalysis (Matthews, 1988). All of the three BoNTs assayed here show a single high affinity binding site for zinc with dissociation constants in the 40–100 nM range and multiple lower affinity binding sites.

Clostridia produce a variety of zinc endopeptidases (Bond and Van Wart, 1984), and, on the basis of our observations, it is tempting to speculate that clostridial neurotoxins have arisen by fusion of a gene coding for a metalloprotease with that of a protein highly specific for binding to the presynaptic membrane. The metalloprotease activity, confined in the L chain, could thus be delivered inside the neuronal cell and act on a specific peptide bond(s) of a component involved in the control of neurotransmitter release.

The possibility that botulinum neurotoxins are zinc endopeptidases readdresses the research aimed at the discovery of the molecular pathogenesis of botulinum. For example, several site-directed mutagenesis experiments can be designed and the product can be tested for activity in *Aplysia* neurons (Poulain, 1988, 1990, 1991) or in permeabilized PC12 cells (Lomneth *et al.*, 1991). Mutation of the glutamic residue of the motif is expected to delete the BoNT-induced inhibition of neuroexocytosis. Indeed, chemical modification of about two carboxyl groups of BoNT serotype A and E causes detoxification (Woody *et al.*, 1989). A lower or nonexistent activity is also expected for the mutants at the two histidines of the motif as well as at the third zinc ligand residue, which we propose to be either Glu²⁶² of BoNT/A (Glu²⁶⁷ of BoNT/B and Glu²⁵¹ of BoNT/E) or Glu³⁵¹ of BoNT/A (Glu³⁵⁷ of BoNT/B and Glu³³⁶ of BoNT/E) on the basis of their conservation among all clostridial neurotoxins.

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Botulinum Type A Toxin: Properties of a Toxic Dissociation Product

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INTRODUCTION

This paper will be devoted to a description of the properties of a substance which makes its appearance when solutions of type A botulinum toxin are brought to pH 7.5. At this pH value the greater part of the toxin forms a diffuse, rapidly sedimenting boundary in the ultracentrifuge, while about 14% sediment much more slowly (7). Partial separation of this slowly sedimenting component having been accomplished in the preparative rotor of the ultracentrifuge (7) it became possible to compare its properties with those of the parent substance. Among the results to be reported here, particular significance must be ascribed to the demonstration that the slowly sedimenting component represents a fully active form of the toxin which is free from the hemagglutinin commonly associated with it.

MATERIALS AND METHODS

Botulinum Toxin

Two specimens of toxin were used in the experiments reported below. These, which will be designated preparations *e* and *f*, were both obtained from a single batch of toxin solution purified by the method of Abrams, Kegeles, and Hottle (1). Preparation *e* was the material precipitated at 5°C. by 0.8 *M* ammonium sulfate and Prepn. *f* was obtained when the ammonium sulfate concentration in the mother liquor from Prepn. *e* was increased to 1.2 *M*. Both preparations were crystalline. In the analytical ultracentrifuge, a 1.6% solution of Prepn. *e* in 0.05 *M* acetate buffer, pH 3.8, formed a sharp boundary of sedimentation constant, s_{20}^0 of 9.5 which accounted for at least 95% of the sedimenting material while the remaining 5% or so appeared as a faint boundary which moved much more rapidly. Under

¹ The material herein presented will form part of a forthcoming Ph.D. thesis by Jack Wagman, Georgetown University, Washington, D. C.

similar conditions a 1.2% solution of Prepn. *f* showed a major boundary of sedimentation constant 12.5, together with subsidiary boundaries with sedimentation constants 17.4 and 11.7 representing somewhat larger proportions of the sedimenting material than the subsidiary component in Prepn. *e*. In each of these preparations, the value of s_{20}^0 for the main boundary was in good agreement with data given by Wagman and Bateman (7) on the concentration dependence of the sedimentation constant for a monodisperse preparation at pH 3.8–4.0, which, by linear extrapolation of $1/s$, yielded a value of 16.5 for s_{20}^0 at zero concentration. When brought to pH 7.5 by dialysis against 0.05 *M* phosphate buffer, the two preparations had the properties summarized in Table I. Since the presence of a slowly sedimenting component was established, in confirmation of the results reported

TABLE I
Sedimentation Data for Fractions of Botulinum Type A Toxin Formed at pH 7.5

Preparation ^a	Concn. ^b g./100 ml.	ϵ rel.			$10^{12} s_{20}^{10}$		
		%			sec.		
<i>e</i>							
1 Original	0.520	15	78	7	7.3	21.8	29.2
2 Upper-half supernatant	0.038	100	—	—	— ^c	—	—
3 Lower-half	0.118	68	32	—	6.9	19.7	—
4 Redissolved pellet	0.586	9	82	9	6.9	21.4	25.4
<i>f</i>							
1 Original	0.400	25	75	—	5.6	21.7	—
2 Upper-half supernatant	0.075	100	—	—	3.7	—	—
3 Lower-half	0.206	91	9	—	4.7	18.7	—
4 Redissolved pellet	1.493	8	92	—	7.4	18.9	—

^a For key to preparations see text, "Materials and Methods."

^b Determined refractometrically, using specific refractive index increment 1.86×10^{-3} .

^c Concentration too low and boundary too diffuse for calculation of s .

by Wagman and Bateman (7) for another toxin preparation, it was appropriate to proceed with fractionation of Preps. *e* and *f* in the preparative rotor.

Ultracentrifugal Fractionation

The toxin solutions, after having been brought to pH 7.5 by dialysis against 0.05 *M* phosphate buffer, were centrifuged for about 3 hr. in the preparative rotor of the model E Spinco ultracentrifuge at an average field strength of $150,000 \times g$. The toxin concentration was about 1%, the volume centrifuged was 10 ml., and the average temperature was 20°C. After centrifuging, the supernatant solution was divided into upper and lower halves, and the gelatinous pellet was redissolved in phosphate buffer, pH 7.5.

Sedimentation and Diffusion Studies

Sedimentation constants were determined using the analytical rotor of the model E Spinco ultracentrifuge. The records were analyzed for sedimentation constants and relative concentrations of components. Diffusion was measured at 3.2°C. in a Claesson cell (4) with the schlieren optical system, and diffusion constants were calculated by the second-moment method.

Electrophoresis

Electrophoretic mobilities were measured in the Klett type of Tiselius apparatus, using the standard form of cell and a bath temperature of 3.2°C.

Other Measurements

Ultraviolet absorption was measured in the Beckman type DU spectrophotometer using the 1-cm. cell.

Specific areas of monolayers of toxin were measured by spreading the toxin solution on 3% NaCl in the small film tray described previously (2) and measuring the area at a film pressure of 13 dynes/cm., using an "indicator oil" end point.

Paper chromatographic amino acid analysis of the hydrolyzed fractions was performed by the method of Housewright and Thorne (5) using both phenol and aqueous ethanol as solvents.

Toxicity for mice was determined by intraperitoneal injection of diluted toxin into 18-20 g. white mice. The toxin was diluted with 1% disodium phosphate buffer, pH 6.8, containing 0.2% gelatin. The volume injected was 0.5 ml. and six mice were used for each dilution. The LD_{50} was defined as the dose, in mg. N, which killed half the mice within 4 days. If dilutions are made in small steps in the region of the end point, the LD_{50} can probably be determined with an accuracy of 10-20%.

Hemagglutination titers were read by the Lush pattern method as described by Burnet (3), using chick red cells.²

Flocculation titers were determined using a crude antitoxic plasma (Lederle).

EXPERIMENTAL

Characterization of Fractionated Toxin in the Ultracentrifuge

The fractionation procedure described above when applied to Preps. *e* and *f* afforded six specimens. The success with which the fractionations were carried out is attested by the result of examining the six specimens in the ultracentrifuge. In both experiments reported in Table I the upper half of the solution contained the slowly sedimenting component, in rather low concentration, virtually free from the rapidly sedimenting material that constituted nearly 80 % of the starting solution. The lower half of the solution contained the slowly sedimenting component con-

² *Erratum.* In the previous paper by the present authors (7) it was erroneously stated that sheep red cells were used for hemagglutinin assay. Chick cells were used throughout.

taminated with 32% of heavy material in one case and with 9% in the other. The solutions made by dissolving the pellets contained 9 and 8%, respectively, of the slowly sedimenting component.

A marked progressive increase in the sedimentation constant of the light component of Prepn. *f* in passing from the upper layer of solution to the pellet provides further evidence of the polydisperse state of this component. There will also be noted in the same experiment discrepancies between the sedimentation constants of the heavy component of the various fractions. It should be mentioned in this connection that at pH 7.5 the solutions become rather turbid and are clarified by centrifuging prior to the performance of an analytical run in the ultracentrifuge or of

TABLE II
Sedimentation and Diffusion Data for Polydisperse Slowly Sedimenting Fraction Formed at pH 7.5 (Dissociated Toxin)
Measurement made in 0.05 *M* phosphate buffer (ionic strength 0.13)
(Solution *f*3, Table I)

Sedimentation constant, $10^{13}s_{20}^w$ (sec.)	4.7 ^a
Diffusion constant, $10^7D_{20}^w$ (sq. cm./sec.)	6.74
Partial specific volume, \bar{V} (assumed)	0.76
Molecular weight, M^c	71,000
Frictional ratio, f/f_0	1.14
Axial ratio, $a/b^{b,c}$	3.4

^a For a solution of concentration 0.206 g./100 ml.

^b For an elongated ellipsoid of revolution.

^c The values of M , f/f_0 , and a/b are provisional since they are based on measured values of s and D and not on extrapolated values.

any other test. Since the rapidly sedimenting component is polydisperse, precipitation would perhaps consist in preferential removal, by aggregation, of the heaviest molecules, thus bringing about a displacement of the distribution curve of sedimentation rates in the direction of a decreased average sedimentation constant. This may account for the tendency of the average sedimentation constant of the heavy component to decrease during the course of an experiment.

Sedimentation and Diffusion Data on Slowly Sedimenting Fraction

Only one of the solutions (*f*3, Table I) was suitable for measurement of a diffusion constant. The results of this measurement, together with the data derived from it, are given in Table II. The average molecular weight, 71,000, for the slowly sedimenting component is probably some-

what too high, since the measured value of the diffusion constant may have been influenced by the presence of 9 % of the heavy component. The value is also open to slight correction when sufficient data become available for extrapolation of s and D to zero concentration.

Other Physicochemical Properties of Fractions

Solution *f*3 contained two electrophoretically resolvable components in phosphate buffer (0.04 *M* phosphate + 0.1 *M* NaCl) at pH 7.4. The mobility of the main boundary was -3.52×10^{-5} sq. cm./v. sec., that of the secondary boundary, corresponding to about 5 % of the non-dialyzable material, was -14.6×10^{-5} . The ultraviolet absorption spectrum of the solution was consistent with the hypothesis that the secondary boundary in the electrophoresis experiment was formed by nucleic acid, since the absorption maximum occurred at 258 $m\mu$, while that of the unfractionated toxin occurred at 278 $m\mu$, in the position characteristic of most proteins.

Chromatographic analysis revealed no apparent differences in the proportions of aspartic acid, glutamic acid, leucine (and/or isoleucine), valine, and phenylalanine present in the various fractions.

The original toxin preparations and the various fractions all formed insoluble films when applied to the surface of a 3 % NaCl solution. The specific areas at 13 dynes/cm. of films formed from the slowly sedimenting material (0.38–0.45 sq. m./mg.) were significantly greater than those formed from the original preparations (0.23 sq. m./mg.) or from the redissolved sediments (0.22, 0.18 sq. m./mg.).

Toxicity, Hemagglutination and Flocculation Titers

The biological tests performed upon the toxin fractions and upon the starting material are reported in Table III. The results of the experiments on Preps. *e* and *f* are concordant in the sense that while indicating that the toxicities of all the fractions are of the same order of magnitude as those of the starting materials, great differences exist between the hemagglutinating activities of the fractions. The hemagglutinin titers given for the fractions from which the rapidly sedimenting component had been largely removed are rough estimates only, but the values suggest that the hemagglutination brought about by these fractions is due solely to the heavier contaminant, and that the slowly sedimenting material is devoid of hemagglutinating activity.

The relatively small differences between the toxicities of the various solutions show some inconsistencies, for the data on Prepn. *e* would sug-

gest that the toxicity is equally distributed throughout the protein nitrogen of the various fractions, while in the case of Prepn. *f* it would seem that the slowly sedimenting component is significantly more toxic than the starting material or the sedimented pellet. Inasmuch as the fractionation resulted in a greater degree of separation of the components in the latter case, greater weight should be given to the conclusion suggested by this experiment.

Ramon flocculation tests were performed only upon the material from Prepn. *f*. It will be seen from the results in Table III that when the *Lf* values of the various fractions are expressed in terms of the toxicities of the fractions (in LD_{50} units), there are no significant differences between them. The same is true when the nitrogen content serves as the unit of

TABLE III
Properties of Botulinum Type A Toxin Fractions

Preparation:	<i>e</i>				<i>f</i>			
Fraction: ^a	1	2	3	4	1	2	3	4
Toxicity, $10^{-6} \times LD_{50}/\text{mg. N}$	190	197	186	214	156	220	200	135
Hemagglutination, $10^{-4} \times LD_{50}/\text{ml.}^b$	8	64	32	8	6	66	80	3
Hemagglutination, $10^4 \times \text{mg. N/ml.}$	4.21	32.5	17.2	8.74	3.87	30.0	15.0	2.22
Flocculation titer, $Lf/10^4 LD_{50}$	—	—	—	—	3.0	2.7	2.0	3.4
Flocculation titer, $Lf/\text{mg. N}$	—	—	—	—	470	590	400	460
Flocculation time, min.	—	—	—	—	75	620	90	49

^a Key to fractions: 1 is starting material.

2 is upper half of solution after centrifuging for about 3 hr. at $150,000 \times g$.

3 is lower half of solution.

4 is solution of sedimented material.

^b Chick erythrocytes at 1°C. ; concentrations required for detectable agglutination.

concentration. However, this method of reporting conceals the fact that the slowly sedimenting material flocculated with the antiserum only at relatively high *absolute* concentrations. Fractions *f*2 and *f*3 gave no flocculation when the quantities used were identical with those of fractions *f*1 and *f*4 that had been found to give satisfactory end points. A six- to tenfold increase was needed in order to carry out a satisfactory titration with fractions *f*2 and *f*3, and even under these circumstances the flocculation time was greatly prolonged.

DISCUSSION

Properties of Dissociated Toxin

The experiments reported in this and in the earlier paper (7) have shown that at pH 7.5 type A botulinum toxin exists in the form of two

clearly distinguishable polydisperse components, one of average molecular weight of the order of magnitude of one million, the other at least one order of magnitude smaller. Although the range of molecular weights existing about these two average values is unknown, it is probably justifiable to assume that there is no significant overlap, and to treat the two components as though they were distinct entities with certain properties in common and with certain important differences.

In the following discussion the rapidly sedimenting polydisperse component at pH 7.5 will be referred to as the "*complex toxin*," the slowly sedimenting polydisperse component as the "*dissociated toxin*," while the material of molecular weight of about one million that exists below pH 4 in monodisperse form in the best preparations (and with some paucidispersity in others) will be designated "*paucidisperse toxin*." These names have the merit that they avoid any commitment as to whether the polydispersity of the "complex toxin" is one of molecular shape or of size.

In certain respects the complex toxin and the dissociated toxin appear to be alike. They were not distinguishable on the basis of their contents of several amino acids. The electrophoretic mobility of the dissociated toxin, -3.52×10^{-5} sq. cm./v. sec. at pH 7.4, is consistent with the pH mobility curve recorded by Abrams, Kegeles, and Hottle (1) for what was then regarded as the pure toxin, while the presence of a secondary boundary which can be attributed to free nucleic acid explains the observed differences between the ultraviolet absorption spectra of the various fractions prepared by us. The nucleic acid is clearly in the uncombined state and its presence in the "dissociated" or monomeric form of toxin preparation probably means that a trace of nucleic acid attached to the original material at pH values below 4 becomes released at pH 7.5.

In toxicity, the dissociated toxin resembles the parent material, although perhaps significantly more toxic. On the other hand, the hemagglutinin titers indicate that the type A toxin can exist in a form which is not capable of agglutinating red cells. Whether, conversely, the agglutinin can be obtained in nontoxic form has not been shown by the present experiments. This possibility seems however to be indicated by the observation (6a) that the toxicity of toxin solutions is not decreased when the solutions have been used to agglutinate red cells. Recently Lamanna and Lowenthal (6b) have brought immunological evidence that botulinus antitoxin preparations contain two components, an antitoxin and an antihemagglutinin, of which the latter is relatively nonspecific. Finally, unpublished experiments in this laboratory by M. S. Davis and P. A.

McCaffrey show conclusively that the hemagglutinin is removed from toxin solutions when red cells are agglutinated, without bringing about any measurable change in the sedimentation diagram of the toxin.

The relationships between the various forms of the toxin are by no means clear. The toxicities/mg. N being almost equal, it would seem reasonable to suppose that the paucidisperse form found at pH values below 4 consists of an assembly of the smaller toxin molecules which at some stage during the course of physiological action of the toxin becomes transformed to the complex toxin and then completely dissociated into its components; these may tentatively be identified with the fundamental toxic units, although the experimental evidence does not preclude the possibility that the toxic unit is still smaller than the molecule of dissociated toxin. The complete dissociation has not yet been observed in the laboratory; at pH 7.5 it occurred only to the extent of about 20 % under the conditions of our experiments, even when several days were allowed for the process. This may represent complete dissociation of 20 % of the original material, the extent of dissociation being limited either by the attainment of an equilibrium which favors the complex toxin, or by other unrecognized factors. Alternatively, it may mean that, on the average, 20 % of the toxic units in the monodisperse toxin are more loosely bound than the remainder; the latter hypothesis would seem to be the more plausible.

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SUMMARY

1. The partial dissociation of botulinus type A toxin at pH 7.5 and ionic strength 0.13 into a slowly sedimenting component has been confirmed.
2. Under the above conditions of pH and ionic strength the sedimentation constant of the undissociated material is about 20 svedberg units, while that of the dissociated portion is about 5 (see Table I). Both are polydisperse.

3. Measurements of sedimentation and diffusion constants of a specimen of the "dissociated toxin" separated in the ultracentrifuge led to values of 71,000 for the molecular weight and 3.4 for the axial ratio.

4. The toxicity of the "dissociated toxin" formed at pH 7.5 is at least as great, per milligram protein nitrogen, as that of the parent substance. The flocculation titer is also the same. The hemagglutinin titer is very low, probably essentially zero.

5. The nonhemagglutinating polydisperse "dissociated toxin" of molecular weight around 70,000 is identified tentatively with the ultimate toxic unit of botulinus type A toxin.

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Separation of Toxin and Hemagglutinin from Crystalline Toxin of *Clostridium botulinum* Type A by Anion Exchange Chromatography and Determination of Their Dimensions by Gel Filtration*

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SUMMARY

Crystalline preparations of *Clostridium botulinum* type A toxin were fractionated on DEAE-cellulose columns with Tris-HCl buffers at pH 8.0. The isolated toxic fraction was free of hemagglutinating activity and contained 5 times the specific activity of the crystalline toxin. The second fraction was a powerful hemagglutinin but was only feebly toxic; it emerged from the column as one or more peaks, under different elution conditions. By rechromatography and immunological tests, the toxicity of the second fraction was shown to be due to contamination with traces of the toxic fraction. By several criteria, the toxic and hemagglutinating components appeared to be at least two different proteins.

Most of the information obtainable from the ultracentrifugal analysis of these substances was also obtained by gel filtration on a Sephadex G-200 column and with much smaller protein concentrations. At physiological pH, the toxic fraction had a molecular weight of 150,000 and a Stokes radius of 48 Å; these dimensions were in agreement with those established *in vivo* by other investigators. The hemagglutinin appeared to exist in three forms of aggregation with molecular weights of 290,000, 500,000, and 900,000.

Since the toxin of *Clostridium botulinum* type A was obtained in the crystalline form (1), its homogeneity has not been seriously questioned. However, observations (2, 3) showed that the crystalline toxin was not only highly toxic but also hemagglutinating, and that the hemagglutinin could be dissociated from the toxin without loss of toxicity (4) and with a two- to three-fold increase in specific activity (5). In addition, in the ultracentri-

fuge under certain conditions, the toxin appeared to contain at least two distinct components with significantly different $s_{20,w}$ values (5-7). This evidence did not change the notion that the crystalline toxin is a homogenous protein of molecular weight 900,000, since the multiplicity of components and the two different biological activities were ascribed to the properties of the same molecule in different states of aggregation (5).

Schantz, Stefanye, and Spero (8) attempted to fractionate crystalline toxin on a DEAE-cellulose column but obtained material with no greater toxicity than was observed in the crystalline toxin. Gerwing, Dolman, and Bains (9) have reported isolation of type A toxin of molecular weight 12,000 obtained from crude culture filtrates by 50% $(\text{NH}_4)_2\text{SO}_4$ saturation with subsequent purification of the isolated material on a DEAE-cellulose column at pH 5.6. Neither of these studies discussed the possible presence of the hemagglutinating activity in their purified material.

Observations made in our laboratory (10, 11) raised strong doubts as to the homogeneity of crystalline toxin. In a preliminary report (12), we demonstrated that crystalline toxin can be chromatographically resolved into two fractions, α and β , which exhibit different physical, chemical, and serological properties.

This communication describes the resolution of the crystalline toxin into at least two different proteins, a toxin and a hemagglutinin. The latter, in turn, is separable into three components of different molecular dimensions. Also reported is the estimation of the molecular dimensions of the isolated fractions of the crystalline toxin, particularly the toxic moiety α at low protein concentration and at approximately physiological pH. Finally we consider whether the α fraction represents the molecules that appear at the myoneural junctions where the toxin acts.

MATERIALS AND METHODS

Crystalline preparations of *C. botulinum* type A toxin were obtained through the generosity of Dr. E. J. Schantz, Fort Detrick, Frederick, Maryland. The crystals, stored in 0.9 M

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(NH₄)₂SO₄, were collected by centrifugation and dissolved in an appropriate buffer at room temperature. The dissolved toxin was freed from (NH₄)₂SO₄ by dialysis for 16 hours at 4° against 100 times the volume of the buffer to be used later in chromatography. Any residual turbidity was removed by centrifugation. The protein concentration and the index of purity of the toxin samples were determined as suggested by Schantz (13).

The sources of the other proteins used in this study were as follows: cytochrome c (horse heart, Sigma Type III, lot No. 1148-7150); bovine serum albumin (College of American Pathologists, Chicago, Ill., lot No. 18); glyceraldehyde-3-phosphate dehydrogenase (Calbiochem, lot No. 501536); aldolase (rabbit muscle, Calbiochem, lot No. 54588); catalase (Worthington, CTR 5665); β -galactosidase (14) (Dr. E. J. Steers, Jr., National Institutes of Health, Bethesda, Maryland). Blue dextran and Sephadex G-200 were obtained from Pharmacia Chemical Company Piscataway, New Jersey.

Three different batches of DEAE-cellulose (Cellex-D, Bio-Rad Laboratories) of exchange capacity 0.61, 0.70, and 0.78 meq per g were combined for use. The material was soaked in 1.0 N NaOH for about 2 hours, washed repeatedly with water to the pH of water, suspended in 1% HCl for an hour, and washed again with water to the pH of water. This cycle was repeated until the DEAE-cellulose changed from orange-yellow to white. After the last wash cycle, the cellulose was washed once with a buffer and suspended in the same buffer until ready for use. Columns of 0.9 cm were packed to a height of 32 to 34 cm with the DEAE-cellulose and equilibrated with about 500 ml of the desired buffer for about 16 hours. The bed height was adjusted to 30 cm (column bed volume ~ 28 ml), and glass wool was layered on top. All ion exchange chromatography was performed at 24 ± 2°. The columns were eluted at atmospheric pressure at flow rates of 25 to 35 ml per hour. The eluate was collected in 2.8-ml fractions with a Gilson fraction collector.

Preparation of Sephadex gel and packing and operation of the columns were carried out essentially following Andrews' method (15). Protein samples in 1 to 2 ml were layered over a thin glass wool layer covering the gel bed. Column flow rate did not exceed 30 ml per hour. The columns were operated at 24 ± 2°.

Buffers were prepared by titrating the acidic and basic conjugates of the same molarity to the desired pH; e.g. 0.067 M citrate-phosphate buffer, pH 5.6, was prepared by titrating 0.067 M Na₂HPO₄ with 0.067 M citric acid to pH 5.6. Phosphate buffer was prepared with Na₂HPO₄ and NaH₂PO₄ solutions. The concentration of Cl⁻ in Tris-HCl buffer was determined by titrating Cl⁻ against standard AgNO₃ solution with K₂CrO₄ as indicator. A calculated amount of Cl⁻ as NaCl was added to Tris-HCl buffer for linear and stepwise gradient elution.

For long Sephadex columns, 0.05 M Na₂CO₃-NaHCO₃ buffer, pH 9.2, was adjusted to 0.5% NaCl with solid NaCl. This caused a slight drop in pH, which was readjusted to pH 9.2 with Na₂CO₃ solution. Other buffers of pH 7.2, 7.5, and 8.0 were prepared by titrating 0.05 M solutions of Tris and HCl, both of which contained 0.1 M HCl.

The Stokes radius of the toxin molecules were determined by use of the equation

$$K_D = \frac{V_e - V_0}{V_i} = \left(1 - \frac{a}{r}\right)^2 \quad (1)$$

$$\left[1 - 2.104 \left(\frac{a}{r}\right) + 2.09 \left(\frac{a}{r}\right)^2 - 0.95 \left(\frac{a}{r}\right)^3\right]$$

describing the molecular sieve action of a Sephadex G-200 column (16, 17). K_D is the distribution coefficient of the eluted material, V_e is the elution volume of that material, V_0 is the void volume, and V_i is the effective internal volume of the column. V_0 and V_i were established according to Rogers, Hellerman, and Thompson (18) and Habeeb (19). The Stokes radius of the eluted molecule is denoted by a ; the effective pore radius of the gel, by r .

The volume of the eluate collected from the Sephadex column (2.5 × 50 cm) in every fourth tube was measured, and the mean effluent volume per tube was calculated. The variation in volume from tube to tube was never more than ±2.5% from the mean volume of 2.5 ml. The V_e of a solute was estimated from the elution diagram by extrapolating the sides of the solute peak to the apex.

Protein concentrations of the fractions were determined spectrophotometrically in a Zeiss PMQ II spectrophotometer at 278 mμ and by measuring the fluorescent intensity in the Aminco-Keirs spectrophotofluorometer at 285 mμ excitation and 350 mμ fluorescent wave length. Nitrogen determination was carried out by a modification of Nessler's method (20). The nitrogen content of this protein was tentatively taken as 16.25%. In cases of low amounts of protein, where Beer's law at 278 mμ failed, an excellent linear relationship was found between protein concentration and fluorescent intensity. This relationship held to one-fourth the limit of protein concentration measurable by absorbance and could be extended further by increasing the sensitivity of the apparatus. Thus, protein concentrations as low as 4 μg per ml could be measured. Amounts of protein eluted as individual components were estimated by adding fluorescent intensity and the volume of each fraction.

For rechromatography experiments, effluent containing the peaks from the first chromatographic run were dialyzed for 4 or more hours, with hourly changes, against a 10-fold volume of the buffer to be used. Linear gradients were generated by allowing 130 ml of buffer with high salt concentration to mix with 130 ml of the buffer used for equilibrating the column. Both vessels were of the same dimensions and were placed at the same horizontal level.

The toxicity was assayed for the number of minimal lethal doses by injecting 0.1 ml of the eluate intravenously into white mice weighing 20 g and noting their survival time (21). The hemagglutinating activity of the eluted samples was titrated by two methods. In a rapid test, a drop of human red blood cells in suspension was mixed with a drop of test material on a microscopic slide, and the reaction was noted with or without the aid of a microscope. Semiquantitative estimation of the potency of the hemagglutinins in the test solution was provided by adding 0.5 ml of a 2.0% suspension of human red blood cells in 0.9% NaCl solution to 0.5 ml of serially diluted eluates. The mixtures were incubated at 37° for 60 min and at 4° overnight. The most dilute test sample which caused cell clumping was recorded.

Ion Exchange Chromatography of Crystalline Toxin of C. botulinum Type A—In preliminary experiments, the toxin in 0.067 M citrate-phosphate buffer, pH 5.6, was applied on DEAE-Sephadex or DEAE-cellulose columns (0.9 × 30 cm) previously equilibrated with the same buffer. Immediately after the elution of 1 column volume, a sharp peak emerged. Further elution with the same buffer or with buffer of increased molarity yielded no more protein. The first indication of separation of crystalline toxin was noted when 0.05 M phosphate buffer, pH 6, was

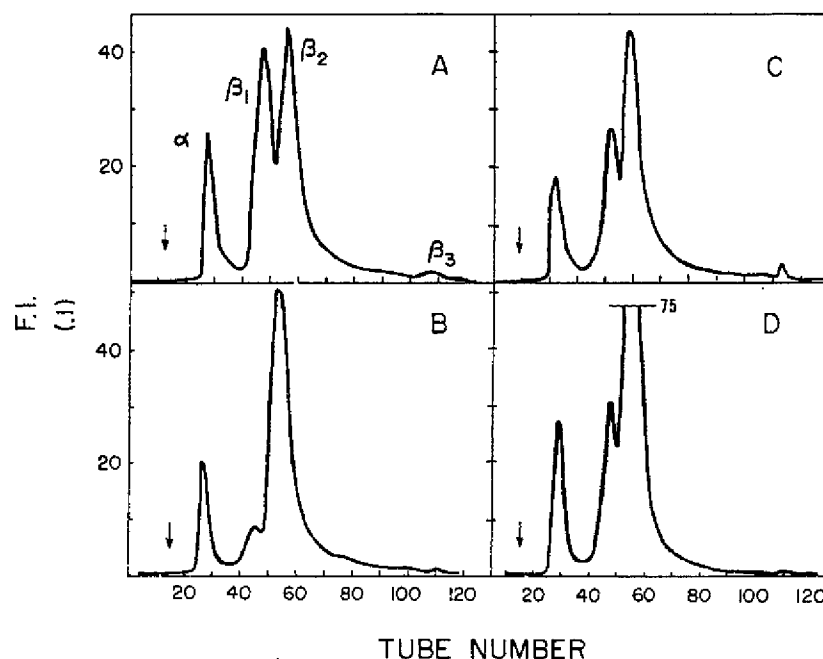


FIG. 1. A through D, chromatography of four different batches of *C. botulinum* type A toxin isolated and crystallized at four different times between a period of $\frac{1}{2}$ to 2 years. DEAE-cellulose columns, 1×30 cm, were equilibrated with 0.15 M Tris-HCl buffer, pH 8. After 4.5 mg of toxin were applied, columns were eluted with the starting buffer until linear gradient elutions were

started at the point indicated by arrow. Fraction size, 2.8 ml per tube; 0.1 is the instrument setting for fluorescence intensity (F.I.) measurement. The linear gradient was generated by allowing 130 ml of 0.15 M Tris-HCl buffer, pH 8, containing 0.5 M Cl^- , to flow into a mixing chamber containing 130 ml of the starting buffer. Elution was complete with 260 ml of salt gradient.

used with a linear NaCl gradient for elution. Clear separation of crystalline toxin into two components was achieved at a higher pH (12). The use of DEAE-Sephadex became impracticable because of significant shrinkage of the column bed in the presence of a salt gradient; therefore, DEAE-cellulose was used subsequently.

Toxin, 4.5 mg was applied on a DEAE-cellulose column, 0.9×30 cm, equilibrated with 0.15 M Tris-HCl buffer at pH 8.0. The column was washed with 2 column volumes of buffer, and a linear gradient was generated with 130 ml of buffer containing 0.5 M Cl^- . With increasing Cl^- concentration, four clearly distinguishable peaks emerged in the eluate (Fig. 1). These peaks were designated α , β_1 , β_2 , and β_3 , respectively. The α peak contained 78 to 80% of the toxic activity (minimal lethal doses) of the stock solution and had about 5 times its specific toxicity ($\sim 1.86 \times 10^6$ LD₅₀ compared to $\sim 3.87 \times 10^7$ LD₅₀ per mg of protein). The β fractions strongly agglutinated red blood cells and were very feebly toxic.

Other batches of toxin, isolated and crystallized from culture filtrates of *C. botulinum* type A grown at different times (ranging from $\frac{1}{2}$ to 2 years apart), were subjected to similar chromatographic procedures. Seven such batches were examined, and all resulted in qualitatively identical profiles, which differed only in the relative concentration of the four components. Fig. 1, A through D shows the chromatographic analysis of four of the seven samples. Crystallization of *C. botulinum* type A invariably resulted in material containing the same four characteristic components.

To establish the identity of the first peak (α) emerging from the DEAE-cellulose, the first half of the eluate, containing the α peak from DEAE-cellulose, was rechromatographed on DEAE-Sephadex. A single peak emerged at the same elution volume

and Cl^- concentration as was obtained originally on DEAE-Sephadex (12). When the procedure was reversed, the α peak obtained from DEAE-Sephadex, when rechromatographed on DEAE-cellulose, also resulted in a single peak.

A clearer separation of the individual components of crystalline toxin was attempted by applying a stepwise Cl^- gradient. The α , β_1 , β_2 , and β_3 fractions emerged at 0.12, 0.19, 0.24, and 0.5 M Cl^- , respectively, in the buffer. NaCl was added to portions of 0.15 M Tris-HCl buffer, pH 8, to bring the net Cl^- concentration to the desired levels. These salt-enriched buffers were used, in order of increased salt concentrations, to elute the fractions of 6.4 mg of toxin applied on a DEAE-cellulose column, 0.9×30 cm. With each of the changes in Cl^- concentration, a peak corresponding to the α , β_1 , β_2 , β_3 fractions emerged (Fig. 2A). The relative concentrations of protein in these components were about 15, 13, 54, and 19%, respectively.

After readjustment to the ionic strength of the starting buffer, the four fractions were rechromatographed with a stepwise gradient as described above. The α peak emerged at the same elution volume as in the original run. Because of small initial yield of the β_1 and β_3 fractions, the respective fractions from two separate runs were pooled for rechromatography. The results obtained with the β_1 , β_2 , and β_3 fractions are shown in Fig. 2, B through D, respectively. The β_1 fraction resolved into four components emerging at the same Cl^- concentrations as were observed with the original toxin, in relative proportions of $\alpha = 5\%$, $\beta_1 = 69\%$, $\beta_2 = 23\%$, and $\beta_3 = 3\%$. The β_2 fraction resolved similarly into four components in positions of α , β_1 , β_2 , and β_3 . The relative proportions of these fractions were: $\alpha = 4\%$, $\beta_1 = 14\%$, $\beta_2 = 52\%$, and $\beta_3 = 29\%$. The elution profile of β_3 showed traces of α and β_1 , while β_2 and β_3 were found in concentrations of 69 and 31%, respectively. On rechroma-

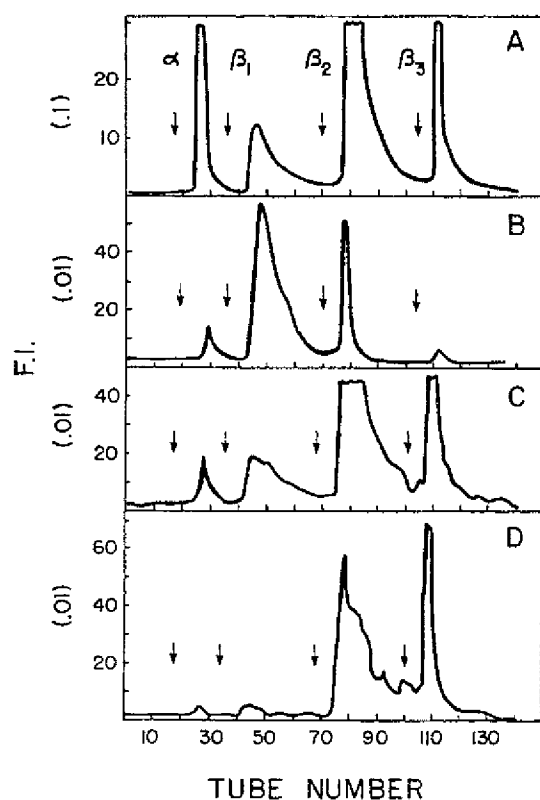


Fig. 2. Separation of the different components of crystalline toxin by stepwise gradient elution and their rechromatography on columns of DEAE-cellulose, 1×30 cm. The columns were equilibrated with 0.15 M Tris-HCl buffer, pH 8.0. Buffers containing 0.12, 0.19, 0.24, and 0.5 M Cl⁻ were applied in the increasing order at the positions marked with arrow. Fraction size was 2.8 ml per tube. Instrument sensitivities for recording fluorescence intensity (F.I.) in Fig. 2, B through D, were 10 times more than in Fig. 2A. A, column loaded with 6.4 mg of toxin was washed with starting buffer until stepwise gradient elution was started. B, elution profile obtained when β_1 , isolated from crystalline toxin (see Fig. 2A), was rechromatographed with conditions as in Fig. 2A. C, elution profile obtained when β_2 , isolated from crystalline toxin (see Fig. 2A), was rechromatographed with conditions as in Fig. 2A. D, elution profile obtained when β_3 , isolated from crystalline toxin (see Fig. 2A), was rechromatographed with conditions as in Fig. 2A.

tography, each of the β fractions yielded some α component. Contamination of the β peaks with the α fraction decreased with the distance of these peaks from the position of α .

In all experiments the α fraction remained highly toxic and free from hemagglutinins, whereas the β fractions were strongly hemagglutinating and still possessed slight toxicity. To determine the nature of toxicity of the β fractions, mice received intravenous injections of each of the three components. These were followed immediately by intraperitoneal injections of rabbit antiserum prepared against the α fraction. All mice treated in this manner survived. Corresponding controls without the anti- α serum died. This was considered as proof that the toxicity of the β fraction was due to the presence of the α fraction.

Sephadex Column Chromatography of *C. botulinum* Toxin—Sephadex G-200 columns can be used for the study of dissociation-association (22, 23) and for the determination of molecular weight (15) and Stokes radii of proteins at very low concentra-

tions (16). The crystalline toxin and its isolated components were therefore examined by this method. About 7.69 mg of crystalline toxin in 3.0 ml of carbonate buffer, pH 9.2, placed on a Sephadex G-200 column (2×114 cm), equilibrated and eluted with this buffer, emerged in two peaks (Fig. 3). The peak emerging first contained 80% of the applied protein and was only feebly toxic, but strongly hemagglutinated human red blood cells. The second peak was slower in eluting, constituted 20% of the protein, and contained most of the toxin. Whereas the specific activity of the starting material contained 7.2×10^3 m.l.d./1.0 absorbance unit, the fast peak contained 4.8×10^2 m.l.d./1.0 absorbance unit and the slow second peak 7.0×10^6 m.l.d./1.0 absorbance unit.

Determination of Molecular Dimensions of Components of Crystalline Toxin—The α , β_1 , β_2 , and β_3 fractions were isolated from crystalline toxin with a DEAE-cellulose column with the use of a stepwise gradient. Aliquots (2.0 ml) of each were chromatographed on a Sephadex G-200 column, 2.5×50 cm, equilibrated and eluted with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M KCl (Fig. 4). The α fraction emerged as a single peak with no visible inflection points on the leading or trailing edge. The effluent following this peak contained no more fluorescent material. Aliquots taken from various tubes under the peak proved highly toxic. The concentration of protein at the apex was 20 μ g per ml. The elution profile of the β_1 fraction showed the existence of at least two major components at $V_e = 102.5$ ml and 117.1 ml, and an inflection area at $V_e = 129.3$ ml. The β_2 fraction eluted as a single peak with $V_e = 103.3$ ml showing no inflection points on the curve. The elution profile of the β_3 fraction had a major peak at $V_e = 105.4$ ml, a shoulder at $V_e = 87.4$ ml on the leading edge, and an irregular trailing edge.

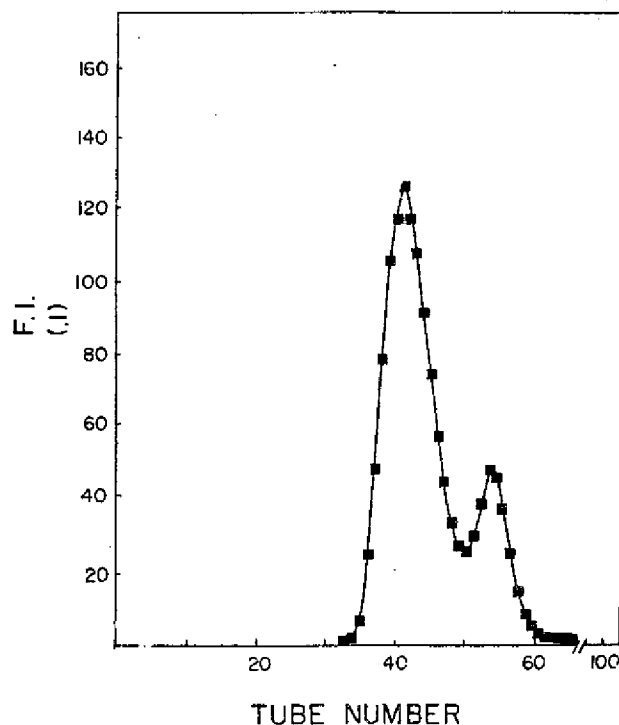


Fig. 3. Gel filtration of crystalline toxin, 7.69 mg in 3.0 ml, on a column of Sephadex G-200 (2×114 cm) with 0.05 M carbonate buffer, pH 9.2, containing 0.5 NaCl. Fraction size, 2.5 ml per tube. F.I., fluorescence intensity.

A group of substances of known molecular weight (cytochrome *c*, bovine serum albumin, glyceraldehyde-3-phosphate dehydrogenase, catalase, β -galactosidase, and blue dextran) were eluted through the same column under the same conditions.

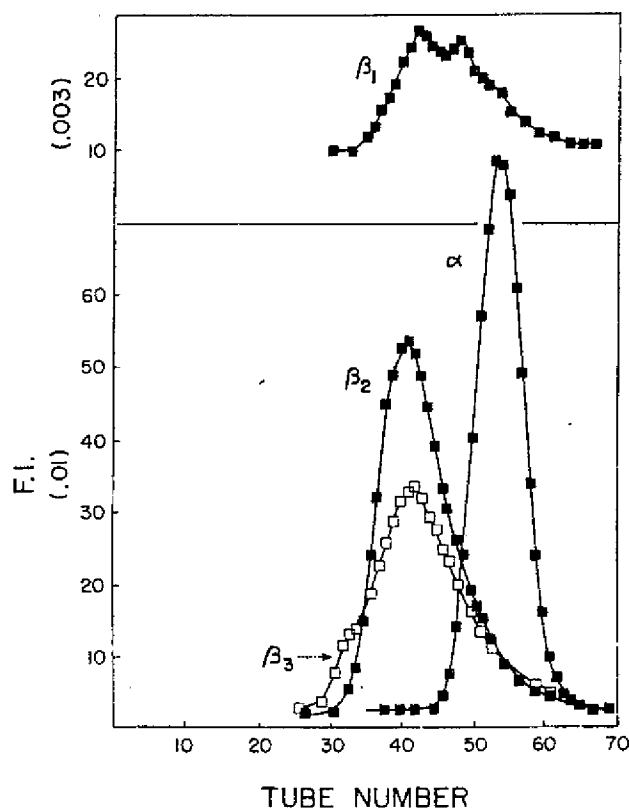


FIG. 4. Gel filtration of α , β_1 , β_2 , and β_3 components, isolated from crystalline toxin by anion exchange chromatography, on a column of Sephadex G-200 (2.5 \times 50 cm) with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M KCl. In the upper part of the figure the elution profile of β_1 was recorded with higher instrument sensitivity than the other three in the lower part. Concentration of α in peak tube was 20 μ g per ml. Similar elution profiles of α were obtained with buffers at pH 7.5 and 7.2 and also with different protein concentrations, so that the peak tube contained 13 or 52 μ g of protein per ml. Fraction size, 2.5 ml per tube.

Each protein eluted as a single peak, with no inflection points; bovine serum albumin dissociated as expected into monomers and dimers. The V_e values of these substances (Tables I and II) plotted against the logarithms of their molecular weights yielded a straight line (Fig. 5). The molecular weights of the α , β_1 , β_2 , and β_3 fractions established from this curve, are shown in Tables I and II and Fig. 5.

The flow rate from the gel filtration column equilibrated with pH 7.2 buffer was too slow to elute all the test proteins. The molecular weight of the α fraction at this pH was therefore not obtained from a calibration curve. Instead, only blue dextran, aldolase, and the α fraction were eluted. Since the K_D value of the α fraction at this pH was similar to that at pH 8, and since the V_e values of aldolase and the α fraction were essentially the same, the molecular weight of the α fraction at pH 7.2 was taken as 150,000. At pH 8.0, with a total gel bed volume of 248 ml, the V_e values for the α component and blue dextran were 133.7 ml and 69.1 ml, respectively. These values for V_e , V_0 , and V_i , substituted in Equation 1, yielded $K_D = 0.360$ for the α fraction. From a similar experiment with pH 7.5 buffer, the K_D of the α fraction was 0.355 ($V_e = 140.6$ ml, $V_0 = 85.8$ ml, gel bed volume = 248 ml), which was similar to the K_D obtained at pH 8.0 (mean deviation $\pm 0.7\%$).

TABLE II
Molecular weight, Stokes radii, and elution volume of toxic and hemagglutinin fractions isolated from crystalline toxin
Clostridium botulinum type A

Crystalline toxin components	Experimentally derived		
	Molecular weight	Stokes' radii	V_e
	$\times 10^5$	$m\mu$	ml
α	1.5	4.79	133.7
β_1	2.9	5.94	117.1
	5.2	7.19	102.5
β_2	5.0	7.13	103.3
β_3	4.6	6.96	105.4
	9.3	9.14	87.4

TABLE I
Molecular weight, Stokes' radii, and elution volume of different proteins and calibration of pore radius of Sephadex G-200 column

Proteins	From literature		Experimentally derived	
	Molecular weight ^a	Stokes' radii	V_e	Pore radius ^b
		$m\mu$	ml	$m\mu$
Cytochrome <i>c</i>	1.24×10^4	1.74 ^c	197.6	22.8
Bovine serum albumin monomer	$6.5-7.0 \times 10^4$		152.5	
Bovine serum albumin dimer	$1.3-1.4 \times 10^5$		132.8	
Glyceraldehyde 3-phosphate dehydrogenase	$1.15-1.45 \times 10^5$	4.13 ^d	142.5	22.2
Aldolase	$1.4-1.5 \times 10^5$	5.0 ^d	133.5	23.8
Catalase	$2.3-2.5 \times 10^5$	5.22 ^c	125.6	22.6
β -Galactosidase	5.4×10^5		100.8	
Blue dextran	20×10^5		69.1	

^a Taken from Andrews (15). Value for β -galactosidase from Craven, Steers, and Anfinsen (14).

^b Mean effective pore radius is 22.85 with a mean deviation of $\pm 2.50\%$.

^c From Ackers (16).

^d From Rogers *et al.* (18).

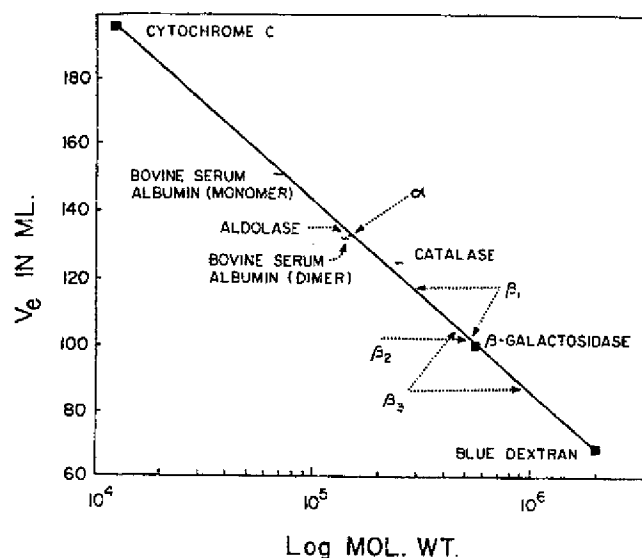


FIG. 5. Molecular weights of proteins as determined by gel filtration on Sephadex G-200 column (2.5×50 cm) with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M KCl. V_e is the elution volume of test substances plotted against their log (molecular weight). The molecular weights of the proteins are: cytochrome c, 1.24×10^4 ; bovine serum albumin monomer, 6.5 to 7.0×10^4 ; bovine serum albumin dimer, 1.3 to 1.4×10^5 ; aldolase, 1.4 to 1.5×10^5 ; catalase, 2.3 to 2.5×10^5 ; β -galactosidase, 5.4×10^5 ; and blue dextran, $\sim 2.0 \times 10^6$. Since β_1 and β_2 each showed presence of two components, the molecular weights of these were determined.

Gel filtration of the β_1 fraction revealed that the two components of β_1 had molecular weights of 2.9×10^5 and 5.2×10^5 , respectively. Similarly, the β_2 fraction had two components with respective molecular weights of 4.6×10^5 and 9.3×10^5 . The molecular weight of the β_3 fraction was 5.0×10^5 . Thus, it appeared that the β_1 and β_2 fractions each contained a component with molecular weight very near that of β_3 (deviations of +4% and -8% from 5.0×10^5), such that β_3 appeared to be a common component of the other two β fractions.

The gel pore radii of the Sephadex G-200 column were established from known Stokes radii and experimentally determined K_D values of cytochrome c, glyceraldehyde-3-phosphate dehydrogenase, aldolase, and catalase. The mean effective pore radius of the gel was $22.85 \mu\text{m} \pm 2.5\%$ (Tables I and II). From Equation 1 the value for the Stokes radius a for the α component was $4.79 \mu\text{m}$ or 48 \AA .

DISCUSSION

The crystalline toxin of *C. botulinum* type A, which for the past 20 years had been considered to be in a high state of purity, was found on chromatographic analysis to contain at least four components. The α fraction proved to be the neurotoxin elaborated by these organisms. The three other components, slightly toxic but containing the hemagglutinating property of the crystalline toxin, were designated as fractions β_1 , β_2 , and β_3 . Since the toxicity of the β fractions could be neutralized *in vivo* with rabbit antiserum prepared against pure α fraction, it was concluded that the toxicity of the β component was due to contamination with α . This was shown by rechromatography of the β components.

Our attempts to purify crystalline toxin with the use of the

chromatographic conditions of Gerwing *et al.* (9) neither separated the hemagglutinins from the toxin nor increased the specific activity of the eluted material. Only at or above pH 6.0 in the presence of a Cl^- gradient did the resolution of the crystalline toxin become apparent. The failure to observe separation of crystalline toxin at pH 6.5 by Schantz *et al.* (8) may be due to the shortness of their column (10 cm). At pH 7.2, pronounced separation of the α and β fractions was achieved (12), although at this pH the α fraction was not completely free of hemagglutinins. Better separation of the α and β fractions was obtained at pH 8.0, at which point the α fraction became free of detectable hemagglutinating activity, and β resolved into three distinct fractions.

Gel filtration was used to examine the homogeneity of the isolated four components and to establish their molecular dimensions. Efforts to dissociate the toxin on a Sephadex G-200 column, 2×114 cm, at pH 9.2 resulted in two components of different molecular weights and different biological activities. Use of longer columns, up to 130 cm, did not improve the resolution. The reason for the incomplete separation of crystalline toxin (see Fig. 3) became evident when overlapping of elution profiles of different fractions was observed (Fig. 4). Occasionally, poorer resolution of the two peaks was noted with different batches of Sephadex. When this occurred, a single peak appeared with a skewed trailing edge. Aliquots from the last half of the trailing edge exhibited higher specific toxicity than the rest of the peak. Andrews (15) explained occasional poor separation of β -galactosidase from blue dextran as being due to differences in the degree of cross linking and, hence, to different water-regaining capacities of different batches of gels.

The symmetry in the elution profile of the α fraction, shown in Fig. 4, remained characteristic during gel filtration at various pH values and protein concentrations (the peak tube contained 13, 20, and $52 \mu\text{g}$ per ml of this protein). The K_D value for the α fraction also remained identical, which suggests that its molecular dimension remained essentially the same. This was in accord with the observations made in the ultracentrifuge (24); under all conditions tested $s_{20,w}$ of the α fraction was 7.25 ± 0.05 .

Although there is no general agreement as to which dimensional parameter of a protein molecule most closely correlates with the gel filtration elution volume, it has been well established by Andrews (15), Whitaker (25), and Leach and O'Shea (26) that the molecular weight may be calculated from elution volume. Andrews (15) showed an excellent linearity between the elution volume and the log of molecular weight of some "well behaved" proteins. According to Ackers (16), Laurant and Killander (27), and Siegal and Monty (28), Sephadex G-200 columns can be used to derive molecular size of a protein molecule. By Andrews' method (15) the molecular weight of the α fraction was found to be 150,000 at pH 7.2, 7.5, and 8.0. By ultracentrifugal analysis the molecular weight of the α fraction, at pH 9.5, was found to be $128,000 \pm 10\%$ (24). In the ultracentrifuge Wagman (7) found a toxic moiety from the crystalline toxin of molecular weight 158,000. The specific toxicity of the component obtained by Wagman was 30% less than that of the starting material (7). In the present study, at low protein concentration and approximately physiological pH, the toxic fraction had a molecular weight of 150,000 and a Stokes radius of 48 \AA . Since many biologically active proteins consist of sub-

units (29), it is possible that the α protein is not a single polypeptide chain.

Although the β fraction produced a single band in Ouchterlony double diffusion tests with rabbit antiserum against crystalline toxin, or antiserum against chromatographically isolated β component (24), it appeared to contain more than one component when analyzed at pH 8.0 on DEAE-cellulose columns. Crestfield, Stein, and Moore (30) found that the aggregated forms of bovine pancreatic ribonuclease could be separated from monomers on ion exchange columns. Thus, the β subfractions were considered as the aggregated hemagglutinin in species of different molecular weights. The validity of this assumption was supported by ultracentrifuge experiments (24) in which, under all conditions of pH and ionic strength used, no more than four components were observed. Of these, one was the toxic α component with $s_{20,w} = 7.2$, and the other three components were of $s_{20,w} = 13, 16$, and 23 , which correspond to the molecular weights of the β_1, β_2 , and β_3 components, respectively. The molecular weights of these three components, estimated by gel filtration, were 290,000, 500,000, and 900,000, respectively. From these molecular weights and from their common biological activities, these hemagglutinin components seemed to be one entity in different states of aggregation. Furthermore, it appeared that the β_2 fraction was a common component of the three fractions because of its invariable appearance upon rechromatography of each of these fractions. The hemagglutinins of the crystalline toxin may have dissociated on the column into distinct fractions under the influence of increasing ionic strength during elution. The amount of material eluted with a stepwise gradient as the β_3 fraction was consistently larger than that obtained with a linear gradient. One possible interpretation is that, after the formation of β_1 and β_2 species under a linear gradient, only a small amount of hemagglutinin remained on the column in the form of β_3 , which eluted much later. In the stepwise gradient elution, the formation of β_2 was suddenly interrupted by the application of 0.5 M Cl^- , and as a consequence the hemagglutinin remaining on the column eluted as β_2 .

Our success in separating the hemagglutinin from the toxic moiety of the crystalline toxin is in agreement with the observations of others, who showed that the hemagglutinin could be removed from the toxin by adsorption on red blood cells (4). Wagman (5) had separated in the ultracentrifuge a hemagglutinin of $s_{20,w} = 14$ from a toxic component of $s_{20,w} = 7$. These authors overlooked the possibility that the neurotoxin and hemagglutinin might be different proteins rather than the same proteins in various forms of aggregation. Wagman (7) found that the $s_{20,w} = 7$ fraction had a lower tyrosine content than the crystalline toxin, and that its absorption spectrum, between 250 and 300 m μ , differed distinctly from that of the crystalline toxin. We have also found this difference in absorption spectra between the α and β fractions and the crystalline toxin¹; this suggests the presence of two different proteins. Furthermore, the α fraction differed from the β fraction in immunoelectrophoretic mobility (12), in Ouchterlony gel double diffusion tests (24), in the ultracentrifuge (24), and in gel filtration analysis. Our preliminary amino acid analysis of the α and β fractions and of the crystalline toxin also showed that the α fraction is significantly different from both the β fraction and the crystalline toxin.

Heckly, Hildebrand, and Lamanna (31) found that the toxin

in lymph which drains the intestinal wall of orally intoxicated animals had a mean $s_{20,w}$ value of 7.9. Zacks *et al.* (32), who studied the site of deposition of *C. botulinum* type B toxin labeled with ferritin granules by electron microscopy, demonstrated the presence of these granules in the primary and secondary clefts of the myoneural junctions of animals that had received injections. A large proportion of these granules were 100 to 150 A apart. Since this spacing was observed only when ferritin-labeled toxin was used for injections, not ferritin alone, they proposed that the intervening space between ferritin granules was occupied by the toxin molecule.

It is recognized that the Stokes radius of a protein may not exactly express its actual dimension. However, if it is assumed that the molecular dimension of the α component obtained by physicochemical means ($s_{20,w} = 7.2$ and 96 A diameter) is close to its actual dimensions, these values agree well with the corresponding values for the *C. botulinum* toxin found in body fluids and at myoneural junctions. Therefore, it is perhaps not too hazardous to speculate that the α fraction of type A toxin, with a diameter of 96 A and $s_{20,w} = 7.2$, can, without further dissociation, penetrate the intestinal wall and reach the receptor sites at the myoneural junctions.

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USE OF CRYSTALLINE TYPE A BOTULINUM TOXIN
IN MEDICAL RESEARCH

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The various toxins produced by *Clostridium botulinum* are extremely potent neurotoxins. Type A toxin (one of the 6 recognized types) is easily produced in deep culture and the first to be obtained in a highly purified crystalline form. It is a high molecular weight simple protein (about 900,000) and dissociates under certain conditions of pH and ionic strength into a protein of about 150,000 molecular weight having the neurotoxin properties and another possessing hemagglutinating properties which appears very important in stabilizing the toxic portion of the molecule. The toxin has the specific physiological action of causing a presynaptic block by inhibiting in some manner the release of acetylcholine at the myoneural junction and producing a flaccid paralysis of the muscle which requires about three weeks or more for recovery.

The work of Scott (1) originally presented at the 84th Annual Meeting of the American Academy of Ophthalmology in San Francisco, California, 1979, and recently published in Ophthalmology (2) has taken advantage of this property of the toxin to treat strabismus in humans by injecting a small amount of toxin under carefully controlled conditions directly into the extraocular muscle pulling the eye out of

alignment. The amount of toxin used in the treatment depends upon the condition of the patient. At the present time the toxin used for this treatment is an ultrafiltered preparation of 0.05 micrograms (μg) of crystalline toxin (116 mouse IP LD_{50}) lyophilized with human serum albumin and saline in small ampoules kept under vacuum. This preparation appears to be appropriate and reliable in every respect for medical use in the treatment of strabismus in humans. Although the specific toxicity of the crystalline toxin of 3×10^7 mouse IP LD_{50} per mg, or 1.5×10^3 LD_{50} for the 0.05 μg in an ampoule, drops more than one log during filtration and lyophilization, this drop is relatively constant from one preparation to another and close to 116 mouse LD_{50} remains in the ampoule. For treatment 0.64 ml or more, depending upon the dose to be given, of sterile saline is introduced aseptically into the ampoule to dissolve the toxin and a water clear solution is produced. A 0.1 ml of this solution, using an electromyographic needle, is injected into the muscle. Experience gained by Scott indicates that about 1 mouse LD_{50} is a starting dose and this is repeated or increased according to the response of the patient. Upon recovery the muscle tends to stay in the proper position and corrected cases, now over 2 years old, have remained so. The maximum time of paralysis occurs 4 or 5 days following the injection, and then gradually diminishes, depending on the dose. The maximum correction of strabismus has been 20 degrees. The maximum follow-up following injection is 6 months. The results after the treatment of 43 cases in humans have been remarkably good and the simplicity of the treatment definitely makes it an alternative to surgery for the correction of strabismus. Details of the treatment are given in publications by Scott (2). One concern regarding this preparation is the presence of detoxified toxin in the presence of active toxin. One injection however would deposit less than one ng of detoxified toxin. This amount seems to be inconsequential and probably insufficient to illicit any antibody production. At least detoxified toxin appears to have no observable effect when injected IP or IV into mice and Dr. Scott has observed no effects on humans.

Although the preparation described above is, for all practical purposes, satisfactory for the treatment of strabismus, the ideal preparation for this treatment, or for any other medical use of the toxin would be one in which the full toxicity was maintained during preparation and on long time storage.

Studies have been undertaken to accomplish the ideal preparation. One of the purposes of this paper is to describe some of the important problems regarding the nature

and properties of the toxin that are involved in its preparation for medical use; that is the use of the toxin as a drug. Botulinum toxin, like other proteins that possess biological activity, such as some enzymes, possesses its extreme toxicity due to its conformational structure (3,4). It is therefore detoxified in solution by heat, various chemicals, dilution to low concentrations, surface stretching and surface drying. To make a preparation suitable for medical use it was necessary to find means to preserve the toxicity and considerations were given to: (a) purity; (b) factors involved in making a reliable and stable preparation; (c) some data on dose response in animals; and (d) sterility of the preparation.

In regard to purity, the crystalline toxin, upon ultracentrifugation at pH 5.6 or below, is a homogeneous substance of constant composition and activity. From the time Lamanna (5) found that the crystalline toxin could be dissociated into toxin and hemagglutinin by treating with red blood cells at pH 7.3 there has been a question about the advisability of using crystalline toxin in physiological research because of the possible effects of the hemagglutinin on the action of the neurotoxin. The separation of the neurotoxin from hemagglutinin by physical means by others (6,7) has pointed out the marked instability of the neurotoxin without the hemagglutinin (8). It is believed that the hemagglutinin is dissociated from the neurotoxin in the body when consumed orally and that the neurotoxin only reaches the site of action. When a solution of the crystalline toxin is injected directly into a muscle both the toxin and hemagglutinin are present. The work of Scott (2) has not indicated any undue side effects of the hemagglutinin when the crystalline toxin was injected into the extraocular muscle. An important point regarding the use of the purified neurotoxin besides its instability is the fact that it cannot be prepared with constant composition and activity.

The stability of the toxin in a preparation for medical use and its long time storage without loss of toxicity is a very important factor if the dose is to be reliable. Crystals of the toxin are stable for several years when suspended in 0.9 M ammonium sulfate solution and refrigerated. Dispensing a suspension of such extremely toxic crystals into units of 10 mg is not practical and cannot be done accurately. Our studies therefore have been directed toward the development of a suitable medium for solution of the toxin that would retain the specific toxicity over a reasonable length of time, perhaps for 2 years. The specific toxicity of the crystalline toxin in solution is 3×10^7 mouse IP LD₅₀ \pm 10% per mg using the white mice available in

our laboratory. The specific toxicity varies with different kinds of mice and the conditions under which the assay is carried out. To get around this variation and consistently produce a uniform preparation the crystalline toxin must be measured by its extinction coefficient of 1.65 for one mg per ml at 278nm in a one cm light path and must have a 260nm to 278nm absorption ratio of 0.55 or less. Solutions of the toxin at concentrations of 2 mg or more per ml in 0.05 M acetate buffer at pH 4.2 are stable for long periods, but dilution to much lower concentrations results in its detoxification within a short period of time. Addition of other proteins such as gelatin or serum albumin greatly helps to prevent detoxification in dilute solution and the addition of gelatin is customarily made when diluting the toxin for the mouse assay. The addition of protein to a solution of the toxin at pH 4 to 4.5 was used for the establishment of a reference standard for the bioassay of toxin in foods and body fluids for the Food and Drug Administration (9). For this preparation a solution of 3X crystalline toxin in acetate buffer at pH 4.2 at a concentration of 2 to 4 mg per ml, accurately determined by its absorbance at 278nm, was diluted to a concentration of 100 ng per ml with a 0.05 M sodium acetate buffer at pH 4.2 containing 3 mg of bovine serum albumin and 2 mg of gelatin per ml. When 0.5 ml of this solution was sealed in 1 ml glass ampoules and stored at room temperature, the toxicity remained at the original level of 2500 LD₅₀ for two years but gradually fell off to about 1000 LD₅₀ or 50% within 5 years. Such a solution should be satisfactory for medical use except for the fact that the toxicity is destroyed upon freezing and no assurance can be made against the possibility that it might be frozen in shipping and handling. Some recent preliminary tests show that citrate buffers at pH 4.8 with gelatin and serum albumin make good stable solutions of the toxin at low concentrations stored at 22°C or frozen at -20°C. After three months storage the toxin at these temperatures and a concentration of 65 ng per ml showed no detectable loss. The toxin is also stable to freezing in succinate or oxalate buffers (4).

Because a lyophilized preparation seemed more practical for a wide variety of conditions we carried out lyophilization of crystalline toxin with gelatin and bovine serum albumin in phosphate buffers at pH 6.2 and 6.8. These buffers were used because freezing did not destroy the toxin. However upon lyophilization there was a certain loss in toxicity which amounted to as much as one log or 90 percent in cases, leaving only 10 percent of the toxin remaining with 90 percent detoxified toxin. Use of the

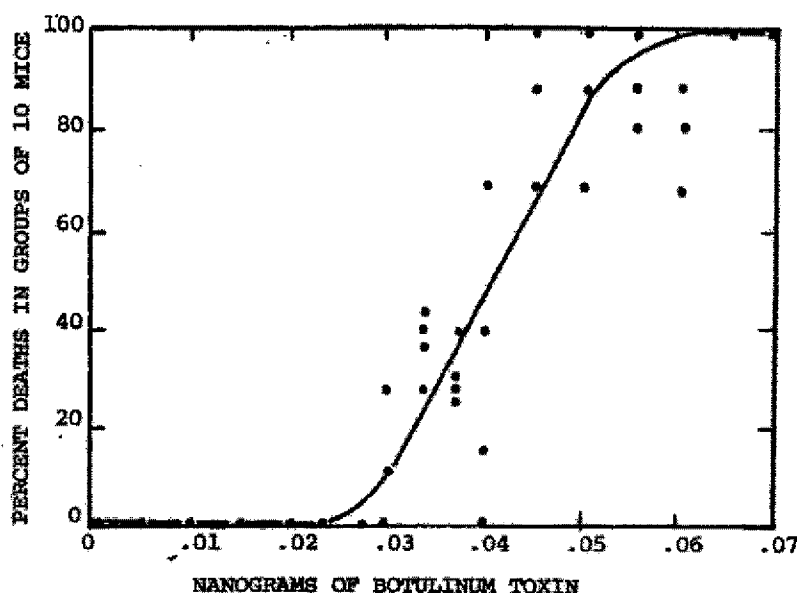


FIGURE 1. Dose response of white mice to type A botulinum toxin. Each mouse challenged intraperitoneally with the dose contained in 0.5 ml of 0.05 M sodium phosphate buffer at pH 6.2 containing 0.2% gelatin. Deaths recorded in a 96 hour period.

toxin in the phosphate-protein buffers would make a good preparation if kept frozen and used immediately after thawing. Standing at room temperature at pH 6.2 or 6.8 results in a gradual loss of toxicity. We are now investigating the use of a variety of different substances along with proteins such as some of the dextrans for stabilization of the toxin during lyophilization.

The toxicity or dose response of crystalline toxin for any medical use in humans must be determined in each particular case. However animal experimentation is indicative of the potency and nature of the toxin. The extrapolation from animal to man cannot be made directly on a weight basis, of course, but the IP dose response in mice, as illustrated in Figure 1, points out the nature of a dose response curve. These data are based on about 350 white mice weighing 18 to 22 grams to increases doses of the crystalline toxin from

0.001 to 0.065 ng contained in 0.5 ml of a 0.05 M sodium phosphate buffer with bovine serum albumin and gelatin to help stabilize the toxin at such dilute concentrations. Each dot on the chart represents the percent dead mice in a group of ten and the combined 35 dots represent the total of three separate trials with 100 to 120 mice on each trial. Some mice showed signs of botulism at about 0.01 ng up to 0.025 ng above which deaths began to appear. Those that did not die recovered within two to three weeks. A dose of 0.065 and above killed all mice in these trials. At least in our mice signs of botulism without death occurred in some mice over a 2 to 3 fold dose and the same was true over the period where death began to occur and where the dose killed all mice.

Other animals have been used. A collection of animal data of various investigators by Smith (10) indicates that 5 mouse LD₅₀ will kill a 500 gram guinea pig by IP injection but 700 LD₅₀ were required by the oral route. Botulism and death occurred in monkeys at 650 mouse LD₅₀ per kg of body weight by the oral route. Swine are very resistant to the toxin and 20,000 mouse LD₅₀ were required per kg by IV injection to cause death and 1.6×10^6 LD₅₀ by the oral route. Dogs are also very resistant to the toxin. In our laboratory 20,000 mouse LD₅₀ of type A toxin per kg by oral route caused no detectable signs of botulism, but 500 LD₅₀ caused signs of botulism by IV injection. These dogs had no antibodies or other toxin neutralizing substances in their blood. Most of the toxin passed through the intestinal tract without absorption and was found in the feces.

One important concern is the amount of toxin to cause botulism in a person. Information on this point can only be obtained, and some has been collected, from accidental cases of poisoning. Estimates from a variety of sources (11) indicate that the dose would be between 0.1 and 1 microgram or about 3,000 to 30,000 mouse LD₅₀, but data collected by Smith (10) from various investigators over the past 60 years indicated a dose as high as 250,000 by the oral route. Most of these data are of little value for cases where the toxin would be injected because they are based on the absorption of the toxin through the alimentary tract and this amount varies greatly from person to person and from one animal species to another. Injection of the toxin and bypassing the alimentary tract makes the response much more uniform. The guinea pig appears to be the most sensitive animal to the toxin by IP injection that we know. If we assume a similar sensitivity for humans and 0.1 ng (2.5 mouse LD₅₀) was injected there would be a safety factor of more than 1000 and for one ng (25 mouse LD₅₀), the highest amount used

by Scott, there would be a safety factor of more than 1000, which is better than most drugs.

Another safety factor to be considered is the sterility of the toxin preparation. Pasteurization by heating toxin solutions buffered at pH 4.2-4.8 in the presence of gelatin or other proteins at 62°C for 30 minutes can be accomplished without detectable loss of the toxicity but heating at 80°C for one minute would destroy practically all of the toxin. Attempts at sterilization of toxin solutions by ultrafiltration in our laboratory caused a 60 percent loss in the toxicity. Sterilization by the addition of bactericidal substances may be the best approach. The two most important functions of antimicrobial preservatives in pharmaceutical products are: (a) protecting the patient from microbial contamination; and (b) preventing loss of toxicity by microbial action. According to the United States Pharmacopeia XVIII multiple dose containers must contain a suitable substance to prevent the growth of microorganisms regardless of the method of sterilization employed. Because multiple dose containers have the advantage of saving medication we are investigating the effect of some of the parabens, organic mercury compounds (thimersal) and substances like chlorohexidine on the toxin during long time storage.

Another point that should be considered here is the name of the toxin, which of course being the most lethal substance known, is scaring indeed to a patient. It is suggested that crystalline type A botulinum toxin to be used in medical practice be called OCULINUM which is derived from the words ocular and botulinum. Other bacterial products, used in medicine, have been designated by names in this manner.

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Clinical and Scientific Aspects of Botulinum A Toxin

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During the past decade, injectable botulinum toxin has been used in ophthalmic and neurologic clinical practice as an unusual and effective form of therapy for involuntary movements and imbalances of muscle tone. This scientific and clinical technology, however, still has significant imperfections, and further development in basic and clinical science is needed. Biologic activity standardization of the toxin using the mouse LD 50 assay during the manufacturing process has been problematic with respect to reproducibility, issues of toxin antigenicity after repeated injections remain unclear, and the most suitable injection methods are as yet undetermined. Such inconsistency can have significant implications on medicinal effectiveness and safety. Because of the pharmacologic importance of the preparation process, the first part of this article reviews the basic principles in drug preparation. Subsequent sections discuss ophthalmic and neurologic clinical studies. Critical review and understanding of toxin preparation can give physicians a basis for critical evaluation of the drug preparation being used clinically.

BASIC SCIENCE OF BOTULINUM TOXIN

Botulinum neurotoxins are produced by certain strains of the bacterial species *Clos-*

tridium botulinum and *Clostridium baratii*.³⁸ The toxins are classified into seven serotypes, A through G. The botulinum neurotoxins comprise a family of pharmacologically similar poisons that block acetylcholine release from peripheral nerves and cause a flaccid paralysis. Type A botulinum toxin is the serotype currently used in clinical practice.

Toxin Purification and Properties

History of Toxin Purification. The first recorded attempts to purify the type A toxin from culture were made in 1928 by Snipe and Sommer⁷⁹ at the Hooper Foundation at the University of California. They showed that 90% to 95% of the toxin could be precipitated from a deep broth culture of *C. botulinum* type A by the addition of acid to lower the pH to 3.5. About 20 years later, Lamanna et al⁵⁰, starting with the precipitated toxin, obtained the type A toxin in crystalline form, and Duff et al²⁴ improved the method that is the basis for the procedures now used to purify toxin for use in the treatment of humans. Lamanna et al⁵¹ discovered that the purified toxin could be separated into nontoxic and toxic components when they found that a nontoxic component precipitated red blood cells, leaving the toxin in solution. Putnam et al⁶⁰ showed that the crystalline toxin moved as a single sub-

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stance in electrophoresis, with a molecular weight of 900,000. Wagman and Bateman⁸⁹ also showed that the toxin moved in the ultracentrifuge as a single substance with a sedimentation coefficient of 19S and a molecular weight of 900,000 at pH 5.6, but at pH 7.3, the toxin component (neurotoxin) dissociated and moved as a much smaller molecule (7S). Later DasGupta and Boroff¹⁷ showed that the neurotoxin could be separated from the nontoxic proteins by column chromatography.

Properties of Crystalline Toxin and Neurotoxin. The crystalline type A toxin contains 16.2% nitrogen and, thus far, has been found to be composed of only biologically active amino acids^{13,80} for both the neurotoxin and the nontoxic proteins. The isoelectric point of the crystalline type A toxin is pH 5.6. Under slightly acidic conditions, pH 3.5 to 6.8, the neurotoxic component of 150,000 Mr (molecular weight) is bound noncovalently to the nontoxic proteins in such a manner as to preserve or help stabilize the secondary and tertiary structure upon which toxicity is dependent. Under slightly alkaline conditions (pH greater than 7.1) and in the blood and tissues of animals and humans, the neurotoxin is released from the toxin complex. The primary structure^{3,85} of the neurotoxin is such that the resulting shape (secondary and tertiary structures) causes highly specific binding and block of acetylcholine release at the myoneural junction.

All of the neurotoxins are synthesized as intact protein molecules with a molecular weight of about 150,000 with low toxicity and are released from the bacterium during culture.⁸² Those from proteolytic (Group I) *C. botulinum* strains are cleaved by extracellular proteases produced by the bacterium into di-chain molecules consisting of a heavy (H) subunit of about 100,000 MR and a light (L) subunit of about 50,000 Mr. The two chains are covalently linked by a disulfide bond and by noncovalent bonds. During cleavage the molecules undergo a molecular change that increases toxicity.¹⁹ Nonproteolytic (Group II) *C. botulinum* strains do not have the endogenous nicking protease, and their toxins are isolated from the culture as single-chain 150,000 Mr molecules. Neurotoxins have been purified for all serotypes except for type G. The H and L chains of the neurotoxin can be separated after reduction by chromatography. The isolated chains are not toxic by themselves but can be recombined under carefully controlled conditions to active

toxin.^{45,54,83} All of the neurotoxins have high specific toxicities ranging from 1×10^7 to 1×10^8 mouse LD50 per milligram of protein.⁸²

Little is known about the nontoxic proteins associated with the neurotoxin except that at least one has hemagglutinating properties. However, all apparently have a role in the stability of the neurotoxin because the type A toxin of high 19S sedimentation rate and 900,000 molecular weight is most stable to digestive and metabolic enzymes.^{63,64} The nontoxic proteins may also help to stabilize toxicity during the injection of toxin into human tissues, but no data are available that directly compare the efficacy of crystalline toxin and isolated neurotoxin. Unless a suitable means of stabilizing the neurotoxin can be achieved, it is apparent that the crystalline toxin (neurotoxin bound to nontoxic proteins) must be the choice for treatment in humans.

Because the toxic properties of the crystalline toxin are due to its structural shape, it is readily detoxified by temperatures above 40°C and on high dilution (milligrams to nanograms), which destroys the shape, particularly at pHs above 7. The loss caused by dilution can be prevented by performing dilution with solutions containing a small amount of albumin or gelatin and by avoiding bubbles, which can lead to stretching and surface denaturation.⁶⁶

Production and Purification of Type A Toxin for Treatment in Humans. The production of type A toxin by injection into muscle nervous tissue necessitates that the production in culture and the purification be performed so that the toxin is not exposed to substances that might be carried through the process in trace amounts and cause undue reactions in the patient. Johnson et al⁴³ have described a process in which the toxin production is carried out in culture medium composed of 2% hydrolyzed casein, 1% yeast extract, and 0.5% dextrose in 12-L carboys. The culture medium contains no animal meat products of any kind, which could present antigenic hazards if slight amounts were carried through toxin preparation. Toxin production is done in this medium with a high toxin producing strain of *C. botulinum* and allowed to ferment at 37°C for 3 to 4 days or until the culture has attained maximum growth and the cells have lysed completely and liberated the toxin into the spent culture.

The toxin is removed from the spent culture for further purification by precipitation with

acid, as indicated previously,⁷⁹ forming a mud-like material about 1/100 of the culture volume containing the toxin. This mud-like precipitate is washed with water and the toxin extracted with salt solution weakly buffered with phosphate at pH 6.5 to 6.8. After another precipitation with acid, the toxin is precipitated with ethanol at -5°C , similar to procedures used for the precipitation of certain blood proteins. The final step in the purification is crystallization of the toxin dissolved in a phosphate-buffered solution at pH 6.8 with the addition of enough 4 M ammonium sulfate to bring the concentration to 0.9 M. Crystallization takes place within a few days at 4°C . During this process, the toxin is not exposed to any enzymes added to assist in the purification or to columns of synthetic resins or dangerous organic solvents of any kind. The toxin will remain stable and active for many years suspended in the ammonium sulfate solution.

The crystalline toxin, when dissolved in 0.05 M sodium phosphate buffer at pH 6.8, has a maximum ultraviolet absorption at 278 nm. The amount of absorption at 278 nm divided by 1.65, the extinction for 1 mg of toxin,⁸⁰ yields the total milligram of toxin in a particular sample and in the entire preparation. From this value the specific toxicity of the toxin per milligram is obtained by determining the LD50 from the serial dilutions of a sample of the toxin that will kill 50% of a group of white mice.⁶⁸ A good and acceptable preparation of the crystalline type A toxin must have a specific toxicity of 3×10^7 mouse LD50 per mg. Another important measurement on a preparation of the toxin used for human treatment is the absorption at 260 nm, which is a measure of the nucleic acid material carried over in the purification. The 260/278 nm ratio is used as one measure or index of purity of the toxin. This ratio for the most highly purified type A toxin has been determined to be close to 0.5, but the value is difficult to attain even under the most careful purification and repeated crystallization of the toxin. Preparations in our laboratory attain a ratio of 0.55 or slightly less and are considered highly purified and should be good for human treatment. Even at a value of 0.6, the amount of nucleic acid-absorbing material would only be 0.08 to 0.1%. This ratio, along with gel electrophoresis analyses, defines the purity of the type A toxin used for human treatment.

For human treatment, the crystalline toxin

must be diluted from milligram concentrations to nanogram concentrations. To stabilize the toxin on such great dilution, small amounts of another protein such as gelatin or albumin are added to the saline solution used for dilution. This diluted toxin solution is filtered for sterility and dried by lyophilization in small vials for dispensing. Although drying helps preserve sterility in case of contamination, it also causes the inactivation of some of the toxin. A final mouse assay must be done for dosage purposes and to meet FDA requirements.

Method of Assay. Because the immunologic properties of the neurotoxin are independent of its toxic properties, the only means to evaluate the effectiveness of the toxin for treatment in humans is by an animal assay for toxicity.⁶⁷ The standardized mouse assay⁶⁸ carried out with the Food and Drug Administration (FDA) reference standard should be used. Assays based on immunologic properties of the toxin yield active as well as inactive toxin and are not recommended for the toxin used in the treatment of humans.

Toxicity in Humans

Botulinum toxin is used clinically by injection of 1 to 300 mouse units depending on the condition being treated. Obviously, it is important that the dose injected be sufficiently low to prevent intoxication. Patients with accidental botulism from toxin-contaminated food have shown symptoms of botulism and, occasionally, have died with 0.1 to 1 μg (100–1000 ng or 3000–30,000 mouse LD50 [MLD50 or U]).^{56,57} Scott and Suzuki⁷⁵ determined that the intramuscular LD50 for juvenile monkeys (*Macaca fascicularis*) was approximately 39 mouse U/kg (approximately 1.25 ng/kg) body weight. Herrero et al³⁹ reported a similar lethal dose of 40 U/kg by intravenous injection in *Macaca rhesus*. No data on the intravenous toxicity for humans are available for these types, but humans are probably at least as sensitive as guinea pigs and expectedly have similar sensitivities as monkeys.

Immunology of Botulinum Toxins

An important factor in the use of botulinum toxin as an injectable protein drug is the elici-

tation of antibodies and possibly other immunities in treated individuals. The minimum dose of toxoid to elicit immunity in humans varies with the individual and toxoid preparation^{1,36} but is probably similar to the immunologic response to tetanus toxoid.³⁶ The amount of toxin needed to elicit antibodies is thought to be greater than the lethal dose for humans. Therefore, antibodies should not develop with current maximum treatment schedules (less than 300 U per treatment). However, if toxin is mishandled during its formulation and drying or during incorrect rehydration by physicians, inactive toxin (toxoid) will be formed, which could promote antibody formation.

Botulinum toxin and antibodies against botulinum toxin can be detected in sera from humans by *in vitro* assays using specific antitoxins. One International Unit (IU) of antitoxin will neutralize 10,000 mouse LD₅₀ except for type E, for which 1 IU will neutralize 1000 LD₅₀. The method currently being developed is the amplified enzyme-linked immunosorbent assay (ELISA) (WH Lee, 1990, personal communication).⁵³ By this method it is possible to measure 10 picograms or less of protein, including botulinum toxin and antibodies to botulinum toxin in human serum. The ELISA method is a linear and quantitative method with a sensitivity comparable to that of the mouse bioassay but differs from the mouse test in that it will detect some but not all forms of biologically inactive but ELISA-active botulinum neurotoxin. The ELISA also can be used to detect hemagglutinin A of the toxin complex. The major limitation in the amplified assay is the quality and behavior of the antibodies. As botulinum toxin therapy becomes more widely used, it may be necessary to have available highly specific and uniform primary and secondary antibodies and possibly Fab' or F(ab)'₂-labeled reagents as second antibodies to lower background color formation.

Use of Other Types of Botulinum Toxin

Seven primary serotypes of botulinum toxin (A-G) are recognized experimentally by the ability of polyclonal antibodies raised against one type to neutralize toxicity in the mouse assay. In patients in whom immunity to botulinum toxin type A develops, it may be necessary to use other serotypes of botulinum

toxin, particularly types that do not immunologically cross-react. Cross-reactions have been detected between toxin types C and D¹⁴ and between types E and F.^{15,21} However, larger quantities of antitoxin for one of the types may be required to neutralize much smaller doses of the other.⁹¹ When prepared against pure toxoided neurotoxin, antisera generally contain a higher titer of antibodies that react with the heavy chain compared with the light chain.^{46,47}

The nontoxin components of some types of toxin complexes have been reported to be antigenically similar to nontoxic proteins of other serotypes.^{47,81} Antibodies obtained from a hemagglutinin fraction isolated from type A toxin complex reacted with nontoxic protein fraction of type B.¹⁹ Nontoxic proteins of types C1 and D,⁸² A and F, and E and F^{64,65} also were reported to be antigenically related or identical.

Evidence has been found that various serotypes of botulinum neurotoxins bind to different "receptors" and with different avidity on nerve termini. Competition binding studies *in vitro* have indicated that different receptors are involved in binding types A and B,^{2,45,91} D,⁵⁸ and F.⁸⁸

CLINICAL PHARMACOLOGY

Botulinum A toxin has demonstrated unique pharmacologic properties as a neuromuscular blocking agent. When injected into striated muscle, the toxin irreversibly blocks the release of acetylcholine and can sustain partial denervation for 3 to 5 months.^{6,7,26,29,72} Observed clinical effects from partial denervation include a reduction of the contractile force of the muscle, a decrease in muscle mass from fiber atrophy, and the reduction of spasm frequency and resting muscular tone. The degree of this effect is dependent on the dose injected, *i.e.*, more atrophy, weakness, and tone reduction result from higher doses. The pharmacologic effect occurs by a three-step process beginning with the binding of a high molecular weight subunit of the toxin to receptors localized at the nerve terminals followed by endocytotic internalization of a low molecular weight subunit of the toxin.^{44,76,77,78} Once internalized, a low molecular weight subunit is responsible for the blockade of exocytotic release of neurotransmitter by a mechanism that remains elusive.^{44,76,77,78} Blockage of neurotransmitter

release begins to occur within minutes to hours. After 2 to 3 weeks, an extrajunctional spread of acetylcholine receptors and acetylcholinesterase at the postsynaptic membrane is observed and indicates denervation.^{9,10,22,23} Also, within several weeks, collateral axonal sprouts develop from preterminal motor axons followed by the establishment of functioning neuromuscular junctions.^{22,23} Apparently, not all collateral axonal sprouts establish neuromuscular junction. Some sprouts lead to "dead ends."^{22,23} Within 3 to 4 weeks, atrophy of muscle fibers can be noted in a pattern similar to that in nerve transections. Figure 1 demonstrates the large degree of fiber size variability 6 weeks after botulinum toxin injection as compared with after saline controlled injections. Collateral sprouting and spreading of acetylcholinesterase has been demonstrated in muscles in humans 5 to 6 weeks after injection with the toxin.^{9,10} After 10 to 12 weeks, evidence of denervation recedes as muscle fiber size variability diminishes and extrajunctional acetylcholinesterase staining decreases.^{10,84} Biopsy specimens of human muscle taken at greater than 6-month intervals after the last botulinum A toxin injection often show no evidence of abnormalities in acetylcholinesterase staining pattern or muscle fiber size variability.^{9,10} The spread of the toxin from the point of injection produces a gradient of denervation within a specific geometric field.¹¹ This spread is not necessarily confined by skeletal or fascial planes because histologic evidence

of denervation has been demonstrated in muscles contiguous to those injected.¹¹

Although botulinum A toxin can cause substantial denervation, there has been no long-term adverse effect on muscle tissue after repeated injections.^{9,11} Fibrosis, contracture, or permanent denervation has not been observed in histologic studies of orbicularis oculi muscle biopsy specimens taken during ptosis surgery from patients who have received repeated injections.

Resistance to the Toxin with Repeated Injections

Because injections must be repeated in most diseases for which the toxin is applied, the possibility of active immunization to the drug is a potential limiting factor. Antibodies have been demonstrated in 7 of 79 patients treated for spasmodic torticollis who have received frequent injections at higher doses (C Hathaway, 1991, personal communication).⁷⁰ Antibodies were not found in a small number of patients treated for blepharospasm for a relatively short period of time.³⁷ Sensitization, however, has occurred in small dose applications with repeated injections, such as for spasmodic dysphonia and occupational hand dystonias (C Hathaway, personal communication). The incidence of antibody formation in long-term therapy for blepharospasm or other applications is still unknown.



Figure 1. Denervation effects on striated muscle. Note the large degree of variability of muscle fiber size.

The long-established bioassay for antibodies to botulinum toxin (mouse assay) is identical to that used in the evaluation of botulinum toxoid effectiveness. Patient serum is mixed with a standardized test dose of botulinum toxin. The test dose is determined against a given quantity of antitoxin (C Hathaway, personal communication). If neutralizing antibodies are present within the patient serum mixed with a botulinum test dose, the mice will survive the lethal injection of a predetermined toxin quantity.

The presence of neutralizing antibodies within patient sera indicates active resistance and has been associated with the loss of effectiveness of repeated toxin injections (E Johnson, personal communication).

INDICATIONS

Strabismus

Strabismus was the first syndrome for which botulinum A toxin was extensively studied. When injected into recti muscles, a temporary denervation results in muscle weakening followed by an alteration of the position of the globe.^{71,74} For example, the patient shown in Figure 2 had undergone four previous operations for strabismus yet remained 45 to 55 prism diopters exotropic. After 5 IU of botulinum A toxin was injected into the left lateral rectus muscle, the deviation was reduced to 15 prism diopters after 2 weeks. This patient demonstrated a substantial short-term benefit of the toxin injection. The long-term result of therapy appears to relate to the patient's fusion potential. The presence of relatively high fusion capability appears to stabilize and sustain alignment and is important to the ultimate success of this procedure and other surgical procedures. In children, an average of 68% improvement in esotropic deviation and 50% in exodeviation was noted based on a study of 356 patients.^{71,74} In adults, a 65% improvement was noted in esotropia and a 61% improvement in deviation with exotropia based on data accumulated by observing 677 patients for an average of 17 months.^{71,74} Repeated injections are common in all categories (45%).^{71,74}

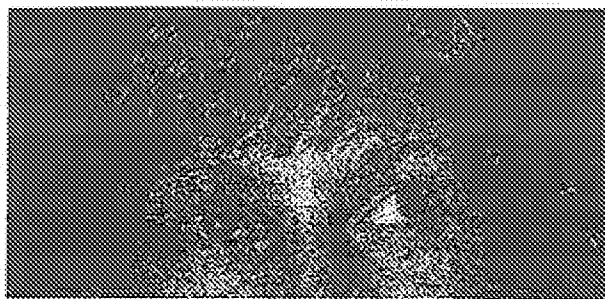
Toxin injections have been used for the treatment of acute and chronic sixth nerve palsies.^{30,49,55,62,73} Although there has been clear benefit as measured by the improve-

ment in horizontal deviation, the effect is generally not sustained unless there is active regeneration of the sixth cranial nerve. Transient medial rectus weakness is temporary and recedes as medial rectus reinnervation occurs. The procedure, however, has been thought to improve the eventual ocular motility by reducing the contracture formation within the antagonistic medial rectus muscle that may occur over a several month period during the early course of sixth nerve palsies. Treatment with botulinum toxin may reduce the number of patients needing vertical rectus muscle transposition surgery,^{30,49,55,62,73} perhaps by reducing this medial rectus contracture. In chronic sixth nerve palsy, the administration of toxin may be helpful in increasing the efficacy of conventional surgery on horizontal deviations.⁶²

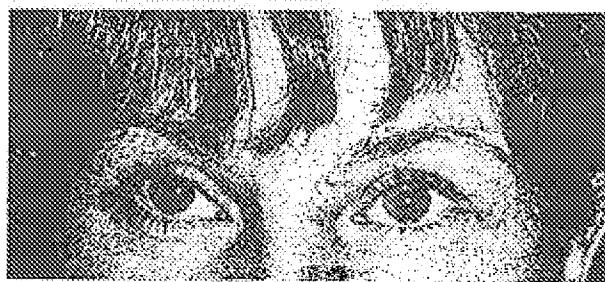
The disadvantages of the application of botulinum toxin for strabismus include the common need for more than one injection and the findings that the alignment is probably not as stable as that achieved with conventional surgery. Excess denervation can result in a transient paralytic strabismus causing diplopia, and vertical deviation and transient ptosis are not uncommon (15% to 20%), and disappointing results have been reported in patients with paralytic and restrictive forms of strabismus.^{71,74}

In summary, it is unlikely that botulinum A toxin will replace conventional surgical procedures for strabismus as the primary form of therapy in the future. However, the application of toxin can be viewed as a helpful adjunct to existing treatments.

Technique. The toxin injection procedure requires the use of electromyographic signal generated from a Teflon coated injection needle. This technique ensures that the needle is clearly within the appropriate muscle tissue. The needle is placed through the anesthetized conjunctiva, and the patient is asked to look to the right and left. As the electromyograph emits the signal as muscle tissue is impaled, the surgeon is assured that the needle is correctly positioned within muscle tissue. This procedure necessitates some knowledge and understanding of simple electromyographic equipment. Because globe perforations have occurred when this technique has been used, informed consent should be obtained. The potential for perforation and the relationship between technique and treatment efficacy underscore the need for training.



A

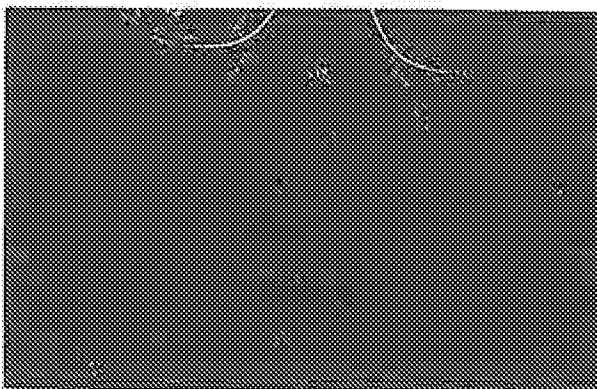


B

Figure 2. A and B, Treatment of severe exotropia with lateral rectus injection of botulinum A toxin. A=Before injection; B=After injection.



A



B

Figure 3. A and B, Involuntary blepharospasm with other involuntary facial movement defines Meige disease.

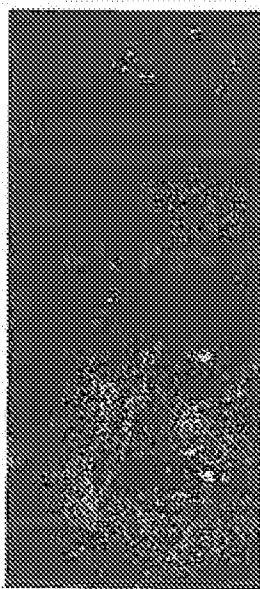


Figure 5. Hemifacial spasm with involuntary synchronous contractions of the muscles innervated by one facial nerve.



Figure 6. Adult-onset spasmodic torticollis with involuntary contractions of muscles rotating and tilting the head and flexing the neck.

Essential Blepharospasm and Meige Syndrome

Involuntary blepharospasm is defined as uncontrolled eyelid closure in the absence of primary ocular disease, such as keratitis or uveitis. The increased blinking or episodic spasmodic contractions of the eyelids usually compromise vision. Frequently, patients seek medical attention when driving or maintaining gainful employment becomes difficult. Involuntary movements are not just limited to the eyelids but may involve other muscles of the head and neck. This condition is termed *Meige syndrome*.^{6,7,26,29,72} Meige syndrome is characterized by involuntary blinking (essential blepharospasm) plus involuntary facial grimacing, frowning, facial contortions, head titubations, spasmodic speech (spastic dysphonia), and various forms of spasmodic torticollis (Fig. 3A and B). This syndrome usually begins with blepharospasm as the presenting sign and progresses to other involuntary movements of the head and neck region in subsequent years. The age of onset is frequently in the fourth and fifth decade, and women are generally more commonly afflicted (60/40). Patients with Meige syndrome have a higher incidence of other family members with facial dyskinesia, bruxism, torticollis, and essential tremor of the head and hand. The condition has been described in identical twins.⁵ Prior therapy for this condition has included the use of neuroleptic drugs and various forms of facial neurectomy and myectomy surgery.^{16,35} Neuroleptic medication often lacks sustained efficacy³⁹ and occasionally results in serious systemic complications, including psychosis, urinary retention, lethargy, and gastrointestinal disturbances.

Although helpful, myectomy surgery is associated with both surgical risks of hemorrhage and potential facial disfigurement. Myectomy surgery also does not completely relieve symptoms of involuntary lid closure and often must be repeated.^{16,35} Facial neurectomy has also been associated with disfigurement and the lack of efficacy, and the need for repeated procedures is not uncommon.^{32,61}

Injections of botulinum A toxin into orbicularis oculi muscle have proved to be effective in controlling various forms of blepharospasm and facial spastic disease.^{6,7,26,29,72} The toxin is injected into 4 to 6 points along the orbicularis oculi muscle and results in partial dener-

vation over a 7- to 14-day period. The beneficial effect is generally maintained for a period of 10 to 14 weeks.^{6,7,26,29,72} Toxin denervation results in a reduction in both the frequency of blinking and intensity of spasmodic contractions. Repeated treatments are almost always necessary to maintain these therapeutic actions. Because denervation of the injected muscle is partial, the patient retains a blink reflex that maintains a precorneal tear film and protects against exposure.

The injection points are outlined in Figure 4. Although these points have been derived empirically based on clinical observation, botulinum toxin injection points appear to have bearing on the response and complication rate. Frueh et al³³ have noted that lower lid injection, when placed medially, causes a higher incidence of diplopia. Presumably, the lower lid toxin injection can diffuse to the anteriorly positioned inferior oblique muscle to induce paralytic strabismus. The upper lid injection also should be positioned close to the lash line along the medial and lateral extent of the upper lid. These upper lid injection positions afford the greatest distance between the orbicularis oculi muscle injections and the muscular portion of the antagonist levator palpebrae superioris muscle. Toxin diffusion into the muscular portions of the levator muscle from upper lid injections can result in transient ptosis. Toxin-induced ptosis may persist several weeks to several months after injection. Multiple injection points over orbicularis muscle have been compared to single motor point injections.⁸

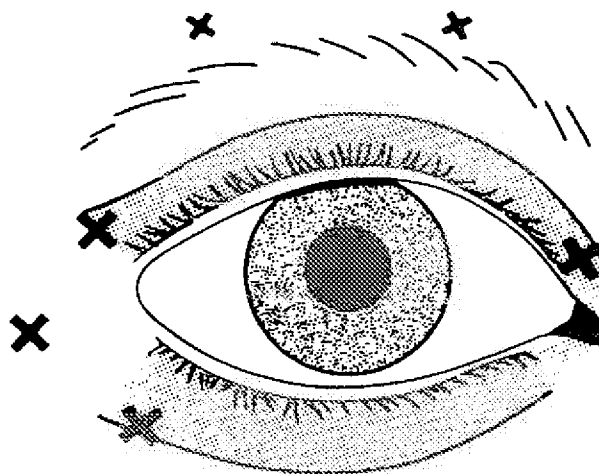


Figure 4. Usual injection points (x) for botulinum A toxin used to treat Meige syndrome.

From clinical observation, it appears the single motor point injections are inferior to multiple injections throughout this muscle. The reason may be that the innervation zone (distribution of neuromuscular junctions) is diffuse for this muscle.⁸ Selective chemodeneration of the orbicularis oculi is the goal in the treatment of blepharospasm, and factors such as anatomic location and the injection of multiple points appear to be important factors in achieving this goal.

To date, patients have been treated with repetitive injections of botulinum toxin for up to 7 years. Analysis of specimens taken during eyelid surgery for myectomy or ptosis does not provide evidence of long-term abnormalities in muscle fiber histology, provided that injections are not administered for 4 to 5 months before a muscle biopsy.¹⁰ Furthermore, no adverse long-term effects have been demonstrated in clinical studies.²⁷

The toxin also has been used successfully in conjunction with myectomy surgery for reducing the degree of surgery necessary to sustain beneficial effects.²⁵

In addition to diplopia and ptosis, complications have included lid malpositions (<2%), lagophthalmos with exposure (<5%), and lid hematoma from injection.

Hemifacial Spasm

Hemifacial spasm is characterized by involuntary synchronous movement of muscles supplied by a single facial nerve. The observed involuntary eyelid closure is associated with other facial muscle contractions drawing the nasolabial fold superiorly, everting the lateral lower lip (Fig. 5). Patients often find this condition extremely disfiguring and functionally incapacitating. Normal facial expressions used in daily communication become interrupted by these involuntary movements. Unlike Meige syndrome, hemifacial spasm is a unilateral form of facial dyskinesia, with bilateral cases being very rare. These patients will often demonstrate weakness on the side in which the involuntary movements are occurring. Electromyographic studies have indicated denervation within the involved facial muscles of these patients.²⁰ A demyelination theory has been proposed to explain the cause of this condition. Based on electromyographic data,⁵⁹ it is thought that demyelination occurring within the intracranial portion of the seventh cranial nerve results in an ec-

topic site of excitation that propagates in an antegrade fashion with ephaptic transmission. This theory is consistent with the clinical findings of facial nerve weakness and synchronous involuntary contractions seen in these patients. Tortuous vessels at the base of the brain pressing on the facial nerve are implicated as a cause of this condition and represent a surgically correctable lesion.⁴²

Treatment with neuroleptic medications has been totally ineffective. Jennetta et al⁴² have proposed the use of posterior craniotomy with decompression of tortuous vessels away from the intracranial portion of the seventh nerve. Although the neurosurgical procedure has proved effective in some instances, patient and physician acceptance of this procedure is poor because the potential complications from neurosurgery are commonly considered unacceptable.

Botulinum A toxin has proved effective in relieving spasms in more than 95% of patients studied.^{6,7} The toxin is injected into the usual points along the orbicularis oculi muscle, as well as into an additional point over the superior portion of the zygomaticus major and minor muscles. The total dose, however, tends to be less than that required for essential blepharospasm, probably because this condition is already associated with denervation. Generally, 10 to 20 IU represents a reasonable starting dose. The average duration of action is 5.1 months, distinctly longer than that for essential blepharospasm or Meige syndrome. Lagophthalmos and exposure keratopathy are also distinctly more common in these patients. Long-term management of these patients has been possible with repeated injections. Other than transient exposure and ptosis, complications are uncommon. Most patients find this approach more acceptable than craniotomy.

Spasmodic Torticollis (Twisted Neck)

Adult-onset spasmodic torticollis^{12,34,39,87} represents a form of cervical dystonia characterized by involuntary contractions with an abnormal increase in cervical muscle resting tone (Fig. 6). Although it usually presents as an isolated syndrome, this disorder may develop in patients diagnosed with essential blepharospasm or Meige syndrome. Spasmodic contractions can lead to posture deformity, cervical pain, decreased active range of motion of the head, varying amplitudes of

head tremors, and noticeable hypertrophy within the sternomastoid, splenius capitis and cervicis muscles, levator scapulae, trapezius, and scalene muscles. Routine activities often become difficult, and persisting disfigurement and chronic pain impair the normal lifestyle.

Prior therapy has included the use of neuroleptic medicines and various forms of denervation surgery. Unfortunately, these therapeutic measures often fail to provide a sustained beneficial response. Complications of surgery include lack of efficacy, weakness from paralysis, and disfigurement.

Botulinum A toxin injections have provided a substantial therapeutic resource for this patient population.^{12,34,39,87} In preliminary studies, relief of pain has been noted in 82% of patients, as well as improvement of posture deformity (70%), increased range of motion (76%), and reduction in visible hypertrophy (85%).¹² The major complication of therapy has been dysphagia.¹¹ Retrospective and prospective clinical studies have indicated, however, that dysphagia may be related to dose and the injection strategy used. This complication is significant because dysphagia has been associated in several patients with upper airway obstruction after swallowing large pieces of meat. The incidence of this complication has been reduced by limiting the dose given to the sternocleidomastoid muscle.¹¹

Because spasmodic torticollis represents a disease of the coordination centers within the central nervous system, multiple muscles of the neck at remote locations are involved. To achieve a higher degree of efficacy, multiple muscle injections are necessary. Prospective clinical data have recently indicated that the technique of multiple injection points per muscle is superior to a single injection per muscle (Table 1). A subclassification of the various patterns of cervical muscular involve-

ment may prove helpful in formulating an injection strategy for each patient.¹² Four subtypes of spasmodic torticollis have been described based on posture deformity and the pattern of dystonic muscle involvement to facilitate toxin administration.¹²

Effective treatment of this disorder requires higher dose administration than in the management of Meige's disease and blepharospasm and has been associated with the development of immunologic resistance to the toxin. Neutralizing antibodies in these patients appear to render the botulism toxin ineffective.

Other Applications

Other diseases with pathophysiology involving involuntary spasmodic contractions that have been studied under clinical protocols include occupational hand dystonia (writers cramp, musicians dystonia),³¹ spasmodic dysphonia,⁵² neurogenic bladder from spasmodic contraction of the external urethral sphincter,²⁸ adductor leg spasms from multiple sclerosis,⁸⁶ and jaw dystonia.⁴ Efficacy has been demonstrated in each of these conditions. Further clinical studies of long-term management of many of these conditions are in process.

CONCEPT OF DENERVATION FIELD AND INNERVATION ZONES

Because the primary mode of action of botulinum toxin involves partial denervation within a given muscle region and because most complications to date have involved undesirable diffusion of the toxin to contiguous regions, methods for focusing the denervation zone would be clinically helpful. Scott⁶⁹

Table 1. Response Rates to Botulinum Toxin for Spasmodic Torticollis Comparing Single- and Multiple-Point Injection Strategies

	SINGLE POINT	MULTIPLE POINT	CHI-SQUARE
Total dose	161 (average)	151	
Pain	15/31	27/31	$P < 0.002$
Posture deformity	13/42	33/44	$P < 0.001$
Range of motion	15/39	33/44	$P < 0.001$
Activity	13/39	29/38	$P < 0.001$
Muscle hypertrophy	27/39	34/44	$P = 0.330$
Tremor	4/17	9/17	$P = 0.080$

proposed the use of antitoxin to contain "toxin jump." Another approach to this problem is the development of an injection protocol that is based on a defined denervation field produced by a unit quantity of the toxin. The denervation field can be defined by measurement of extrajunctional spread of acetylcholinesterase and careful quantization of muscle fiber size variability.¹¹ Such future work may be useful in improving the results obtained with this valuable therapeutic agent. Furthermore, a greater understanding of the distribution of anatomic innervation zones (neuromuscular junction maps) within various muscles may be useful in planning therapeutic injections.

CONSISTENCY OF BIOLOGIC ACTIVITY

As just indicated, containment of toxin effect is important for a safe, effective, and consistent clinical response. Recently, because of variations in biologic activity in the product distributed by the current supplier (Allergan), the FDA has not approved several batches of the material for medicinal distribution. Thus, an interruption in drug availability in the United States has occurred. Because "toxin jump" is a major phenomena resulting in complications (e.g., ptosis, dysphagia, diplopia), consistent standardization of biologic activity is critical in the evolution of this technology.

SUMMARY

Botulinum A toxin has been useful alone or as an adjunct for the treatment of strabismus, facial dyskinesia, spasmodic dysphonia, and spasmodic torticollis. Its efficacy seems to be improved by careful anatomic placement of the injections and the use of multiple injection protocols. Side effects are regional and appear to be related to unwanted toxin diffusion. Defined denervation zones as a function of the unit dose of the toxin and further knowledge of innervation zones of muscles may be useful in the design and the development of more efficacious treatment strategies. Consistent standardization in the biologic activity within the vials represents a current manufacturing problem that necessitates further research.

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